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# IRON OVERLOADING AND HEPATIC VULNERABILITY

L. GOLBERG, M.A., M.B., B.CHIR., D.Sc., D.PHIL., F.R.I.C., AND J. P. SMITH, M.D.

*From Benger Laboratories Ltd., Holmes Chapel, Cheshire, and the Department of Pathology, University of Manchester, England*

We adhere firmly to the conclusion, reached by Sheldon [1] in 1935, that tissue siderosis is not the cause of hemochromatosis but that this syndrome evolves from an inborn error of metabolism, both the tissue changes and the siderosis being directly attributable to this defect.

One experimental approach to this problem is the investigation of conditions leading to increased uptake of dietary iron. By this means, however, the degree of tissue siderosis which can be induced in the lifetime of a laboratory animal is limited. Furthermore, the addition of iron salts to the diet in large amounts brings about unsuspected complications [2] which may completely vitiate the conclusions drawn from such experiments. We have therefore investigated in the adult rat the coexistence of massive parenteral iron overload with a condition which predisposes to excessive iron absorption and the development of tissue siderosis. For this purpose a suitable choice is the antimetabolite DL-ethionine.[3] We have investigated the effect of prolonged oral administration of this agent at a low concentration in the diet concurrently with, and at varying periods after, gross overloading of the rat with parenteral iron. Bearing in mind the predominance of hemochromatosis in males and the often extreme degree of hypogonadism present, a study has also been made of the effects of gonadectomy. Finally, the effect of a diet low in protein and lacking vitamin E has been investigated.

These investigations have reaffirmed our previous view that severe iron overload *per se* does not appear to induce tissue damage. However, our further work now leads to the conclusion that a liver loaded with excessive quantities of iron is vulnerable to the action of toxic agents or deficient diets to a far greater degree than is the case with the normal liver.

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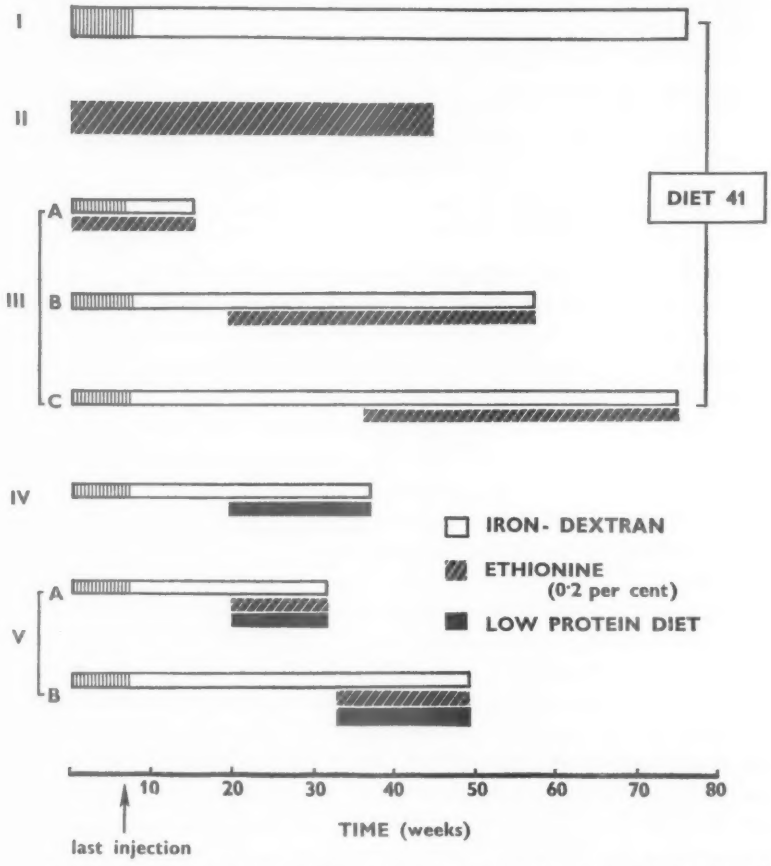
## Material and Methods

Young adult albino rats of both sexes, weighing about 150 gm. (range, 135 to 180 gm. for males, and 115 to 160 gm. for females) were used. Twenty of the male and 54 of the female rats had been subjected to gonadectomy shortly after weaning. The experimental groups, diets and




Text-figure 1. Details of experimental groups, showing the nature and duration of treatments.

duration of treatment are summarized schematically in Text-figure 1. Of the 187 animals finally used, those included in the various groups were as follows (listed in the order: males, females, castrate males and females): Group I: 19, 21, 5, 15; Group II: 8, 7, 0, 3; Group III: 15, 17, 6, 20; Group IV: 5, 6, 4, 6; Group V: 7, 9, 5, 10.

The basal diet was Oxo 41.[4] When DL-ethionine was used, it was applied in solution to the pelleted diet to a final concentration of 0.2 per cent. The low protein diet had the following percentage composition: alcohol-extracted casein (Genatosan), 8; lard, 20; maize starch, 40; sucrose, 21; powdered cellulose, 5; salt mixture (U.S.P. XV, p. 883), 5; vitamin mixture, 0.5; choline chloride, 0.2. Each 5 gm. of the vitamin mixture contained the following: 0.05 gm. each of thiamine, riboflavin, pyridoxine hydrochloride, calcium pantothenate and ascorbic acid; 0.5 mg. each of folic acid and biotin; menaphthone, 0.01 gm.; nicotinic acid, 0.08 gm.; inositol, 0.15 gm.; and *p*-aminobenzoic acid, 0.35 gm.; sucrose to a total weight of 5 gm. Vitamins A and D (3,500 and 300 I.U. per 100 gm. of diet) were added separately in concentrated form immediately before use. It should be noted that no vitamin E was incorporated in the low protein diet.

The routine of iron overloading was the same as that described previously, consisting of 22 doses of 75 mg. of iron per kg. of body weight, administered intramuscularly into alternate hind limbs 3 times weekly.[2,4]

The animals were weighed once a week and finally killed with ether. The necropsy and histologic procedures have been described.[2,4]

## RESULTS

### *Body Weight and Other Changes*

The overall changes in body weight in relation to the initial weights are shown for each group in Text-figure 2. Some groups gained weight steadily, some gained initially but then lost weight, while others lost weight consistently. Loss of weight was for the most part gradual, followed by a sudden decrease which led to the sacrifice or death of the rat.

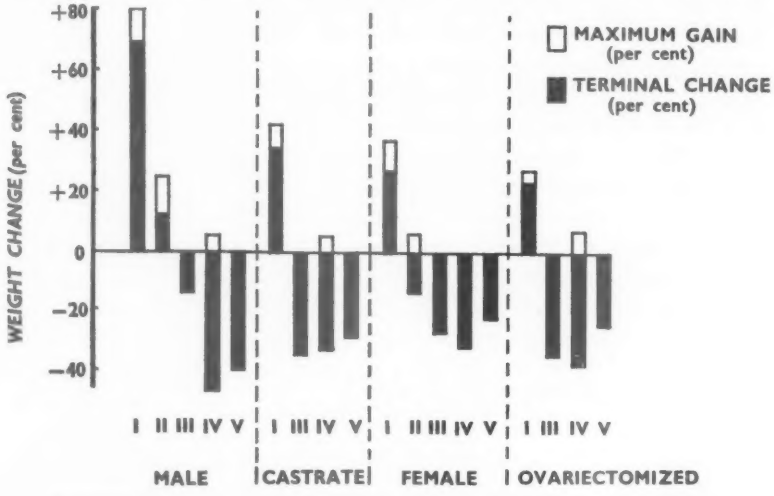
With ethionine (group II) there was inhibition of growth, particularly in females, but on concurrent iron loading (group III) weight loss also set in. By the time the animals were sacrificed they showed thinning of hair, perineal staining and the general unthrifty appearance characteristic of ethionine treatment. Surprisingly, several rats of groups II and III showed some of the alterations attributable to lack of vitamin E, including loss of pigmentation of the incisors with greatly shortened maxillary incisors, paresis of the hindquarters, ataxia and testicular atrophy as well as histologic changes described below.

Low protein diets which induce methionine deficiency rapidly lead to anorexia,[5] and ours was no exception. On the other hand, Kinney, Kaufman and Klavins[3] have shown that such reduction in food intake does not of itself influence the amount of iron in the liver. The deliberate exclusion of added vitamin E from the low protein diet rendered the rats liable to massive hepatic necrosis[6] but was considered necessary in order

to give free play to the "pro-oxidant" action of iron. In fact, at necropsy the animals of group IV and particularly those given ethionine as well (group V) displayed features suggestive of vitamin E deficiency.

### *Lesions in the Liver*

These comprised iron and ceroid pigments in varying amount and distribution, fatty changes and necrosis of parenchymal cells, and the

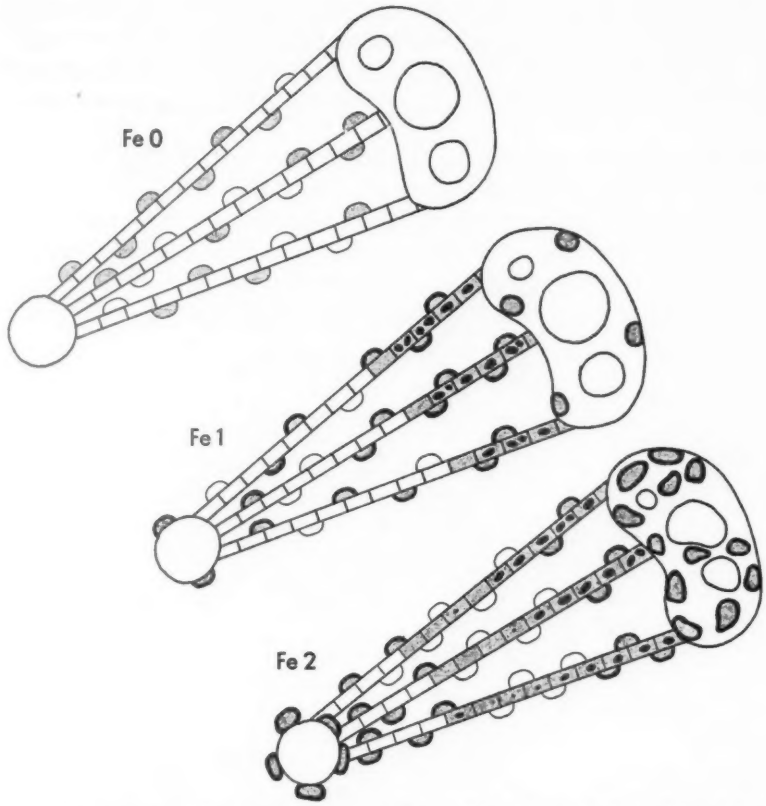



TEXT-FIGURE 2. Average weight changes in the different experimental groups.

eventual development of cirrhosis. The sequence in each experimental group has been divided into stages as follows.

*Group I* (*Text-fig. 3*). During the injection period and for some 15 weeks afterwards iron was found only in the Kupffer cells, many of which were enlarged (Stage Fe0; Fig. 1). During following weeks a slow migration of Kupffer cells toward the portal tracts and central veins led to partial clearing of the sinusoids and the aggregation of small clusters of iron-laden cells in the portal tracts and around the veins. At the same time a little iron appeared in the periportal parenchyma and small amounts of ceroid polymer were found in many iron-laden cells (Stage Fe1; Fig. 2). A year or more after the injections, a more advanced development of these changes (Stage Fe2; Fig. 3) was seen. At no time were there significant fatty changes, and up to 80 weeks no signs of either necrosis or cirrhosis appeared.

*Group II* (*Text-fig. 4*). During the first months (Stage E1; Fig. 4) the livers of ethionine-fed rats showed some disintegration of parenchymal cells about the centrilobular veins and accumulation around



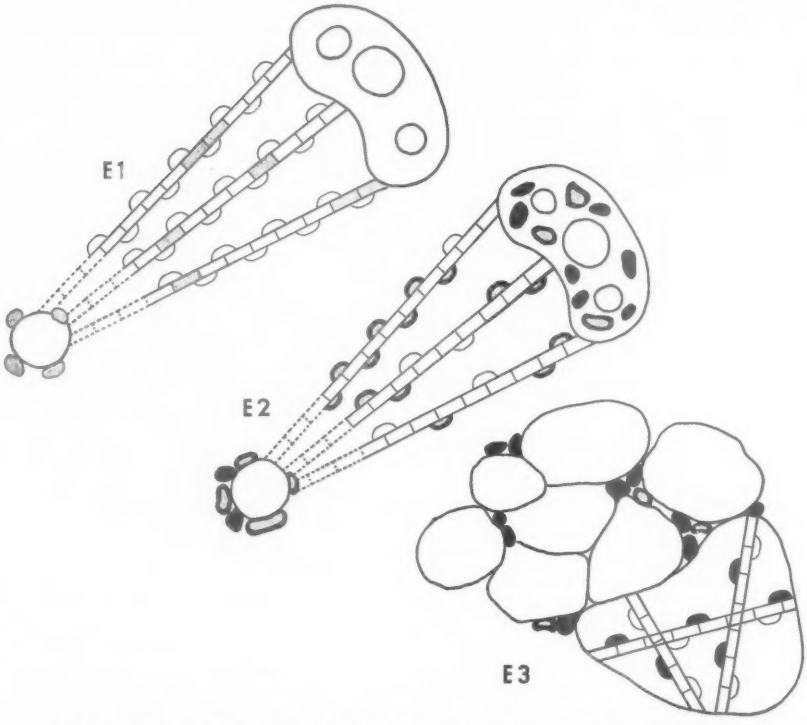

TEXT-FIGURE 3. Diagrammatic representation of time changes in the pattern of iron and ceroid distribution in the livers of iron-laden rats. The stages are indicated by symbols whose full significance is explained in the text. Stippled areas represent iron; solid black areas indicate ceroid.

these veins of iron-containing macrophages. A little iron was seen in some parenchymal cells, but there was no ceroid. At 16 weeks (Stage $E_2$; Fig. 5) much more iron was found in Kupffer cells and in siderophages around portal tracts and central veins; more striking was the presence in these sites of many large ceroid-laden macrophages. It is notable that there was no cirrhosis. All animals surviving 23 weeks showed well-developed cirrhosis (Stage $E_3$; Fig. 6) with small numbers of iron- and ceroid-containing cells in the fibrous septums and ceroid in the Kupffer cells of the new parenchyma. In all these livers there were nodules of bile duct proliferation characteristic of ethionine feeding.

Small amounts of fat, mainly as small droplets, appeared early in these livers, and the amount increased with time though it never became heavy.

In the cirrhotic livers, some lobules contained much fat but others showed none. Necrosis of parenchyma was never seen.

*Group IIIA* (*Text-fig. 5*). Concurrent administration of ethionine and intramuscular iron produced a combination of the changes seen in the




TEXT-FIGURE 4. Time changes in the pattern of iron and ceroid distribution, and the development of cirrhosis in rats given ethionine.

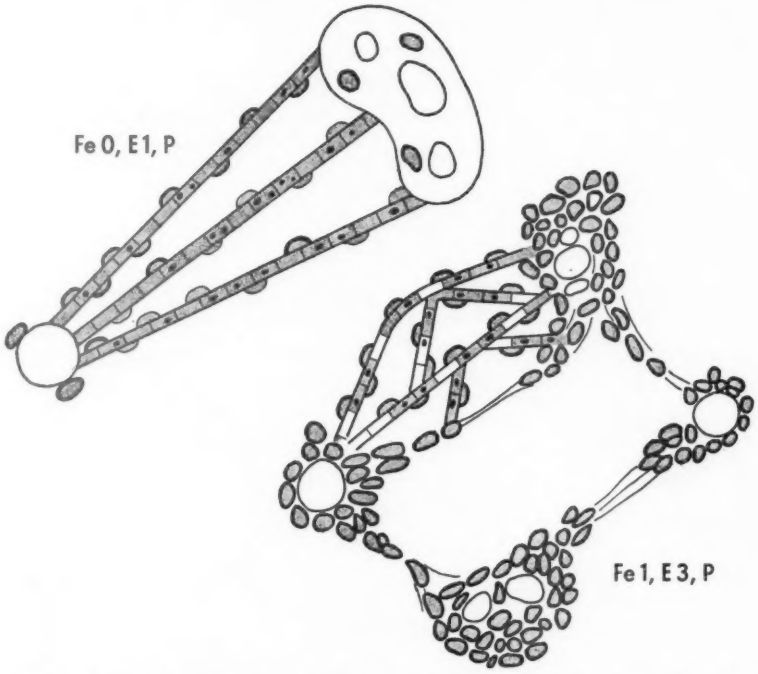
early stages when each was administered separately (Feo and E1). An additional effect was a very heavy, diffuse deposition of iron throughout the hepatic parenchyma represented hereafter by the symbol P. Small to moderate amounts of ceroid were found in most iron-containing cells. The overall picture may be summarized as Stage Feo, E1, P (Fig. 7).

Between 11 and 15 weeks from the beginning of the experiment, early cirrhotic changes were characterized by fine condensations of new reticulin fibers between portal tracts and central veins. Rapid clearing of Kupffer cells from the hepatic sinusoids was followed by their aggregation in large masses around the portal tracts. There was early disorganization of the pattern of the parenchyma: some cells, presumably the original cells, were full of iron, whereas others, presumably newly

formed parenchymal cells, contained no iron. This stage we have represented by the symbols Fe1, E3, P (Fig. 8), though the aggregates of iron were larger than those developing in animals treated with iron alone.

Fatty changes in the parenchyma were similar to those seen in animals of group II.

*Group IIIB* (*Text-fig. 6*). There was essentially no significant difference between the two separate subgroups with intervals of 12 and 29 weeks between iron-loading and feeding with ethionine. The first stage, 19 to 37 weeks after the first injection and 7 to 18 weeks on the diet, showed heavy centrilobular and periportal aggregations of macrophages full of iron and ceroid pigments. Similar pigments were found in most of




TEXT-FIGURE 5. Time changes in the pattern of iron and ceroid distribution, and the development of cirrhosis in rats treated concurrently with iron and ethionine.

the Kupffer cells and diffusely throughout the parenchyma (Fe2, E2, P; Fig. 9).

Some 15 to 20 weeks later an advanced cirrhosis (E4) was present, with very heavy siderophage aggregates—much heavier than could be accounted for by iron loading alone—in the stroma around the new liver lobules. In the new lobules, there was a little iron but no ceroid in many

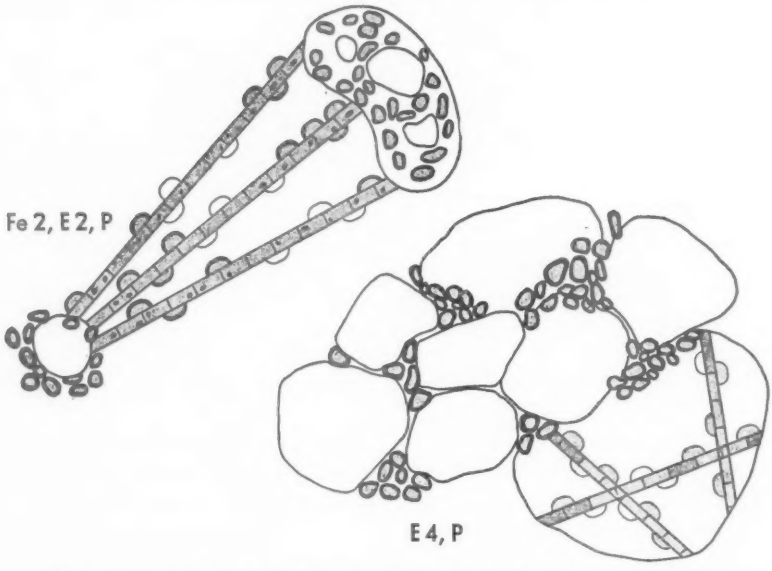
of the parenchymal and Kupffer cells (E4, P; Fig. 10).

Fatty changes in the liver were similar to those in previous groups; in the early precirrhotic stages there was a scanty, very variable random scatter of fat; where cirrhosis was present, fat was heavy in some lobules and absent from others.

*Group IV* (*Text-fig. 7*). All the animals in this group died or had to be killed after only 9 to 18 weeks on the diet. All showed cirrhosis, varying from an early to an advanced lesion, according to the duration of the low protein diet. Very large aggregates of iron- and ceroid-containing macrophages accumulated in the interlobular stroma. A little iron and ceroid were present in some Kupffer cells of the new lobules, but there was no pigment in any parenchymal cells (E4; Fig. 11).

There was much necrosis and atrophy of the livers of these animals and heavy fatty changes persisted throughout.

*Group V* (*Text-fig. 8*). Lesions in this group were the most rapid and severe. Within 6 weeks of commencing the low protein-ethionine diet,

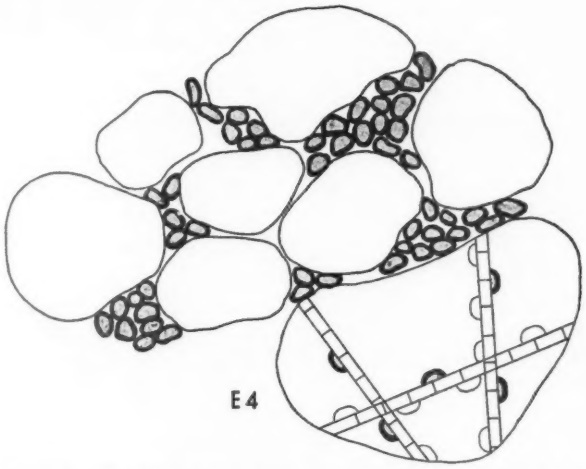



TEXT-FIGURE 6. Time changes in the pattern of iron and ceroid distribution, and the development of cirrhosis in rats treated with iron followed by ethionine after an interval of 20 weeks.

there were large central and periportal accumulations of siderophages and diffuse parenchymal iron, the appearances being similar to the early stage observed in group IIIB but more rapidly produced (Fe2, E2, P). In the next 6 weeks, during which all the animals were sacrificed, a more

advanced cirrhosis developed than was seen in previous groups; the newly formed parenchyma and Kupffer cells contained much iron and ceroid ($E_4$, P and $E_5$, P; Fig. 12).

Severe fatty changes and often extensive parenchymal necrosis were seen in most animals of this group.




TEXT-FIGURE 7. The pattern of iron and ceroid distribution, and cirrhosis in iron-laden rats placed on a low protein diet after an interval of 20 weeks.

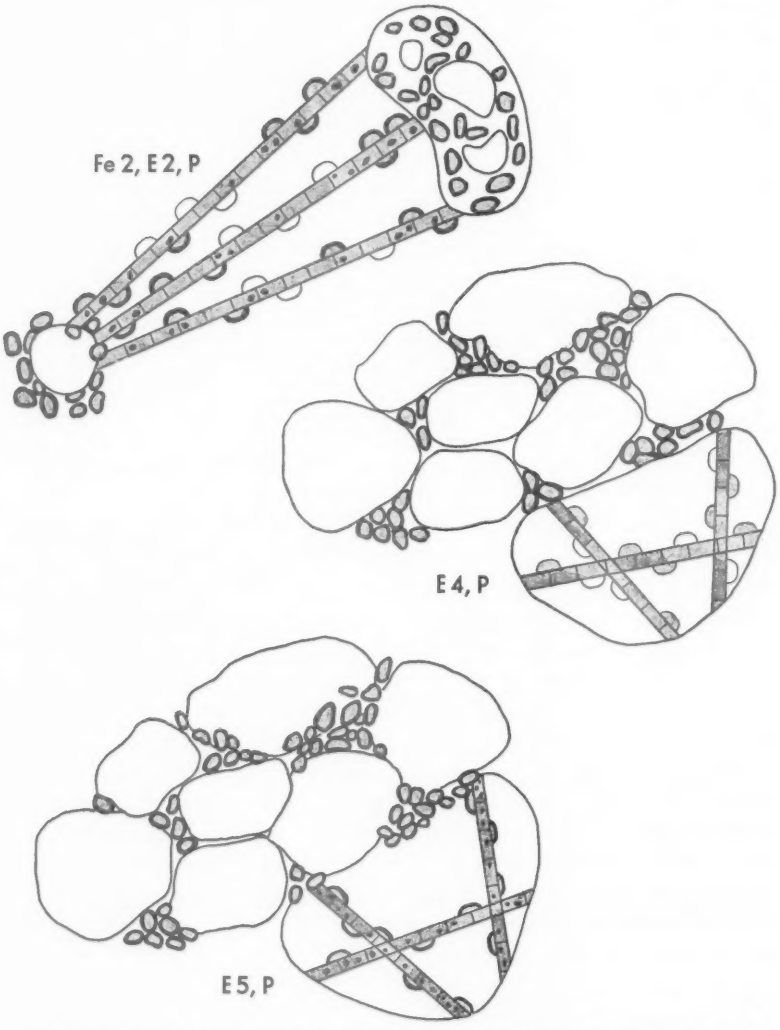
A summary of the liver alterations is shown in Text-figure 9, in which the various stages in each experimental group are plotted against the duration of the experiment. It is readily seen that the concurrent administration of iron and ethionine greatly accelerated the effects of ethionine alone; aggregation of siderophages and cirrhosis developed 7 to 12 weeks after beginning the diet. The effects were still seen, but were less striking when ethionine was fed several weeks after iron loading. These rats, however, lived longer, and an even greater degree of cirrhosis developed, with very heavy iron and ceroid deposition. Iron overloading followed by a diet low in protein induced more severe cirrhosis than ethionine alone or ethionine and iron given concurrently. The combination of ethionine and a low protein diet in iron-loaded animals produced a remarkable acceleration of the whole sequence of changes so that the most severe degrees of cirrhosis and the heaviest concentrations of iron and ceroid developed within a few weeks.

### *Lesions in Other Organs*

The observations in organs other than the liver are summarized in Text-figure 10, in which an attempt has been made to show the relation-

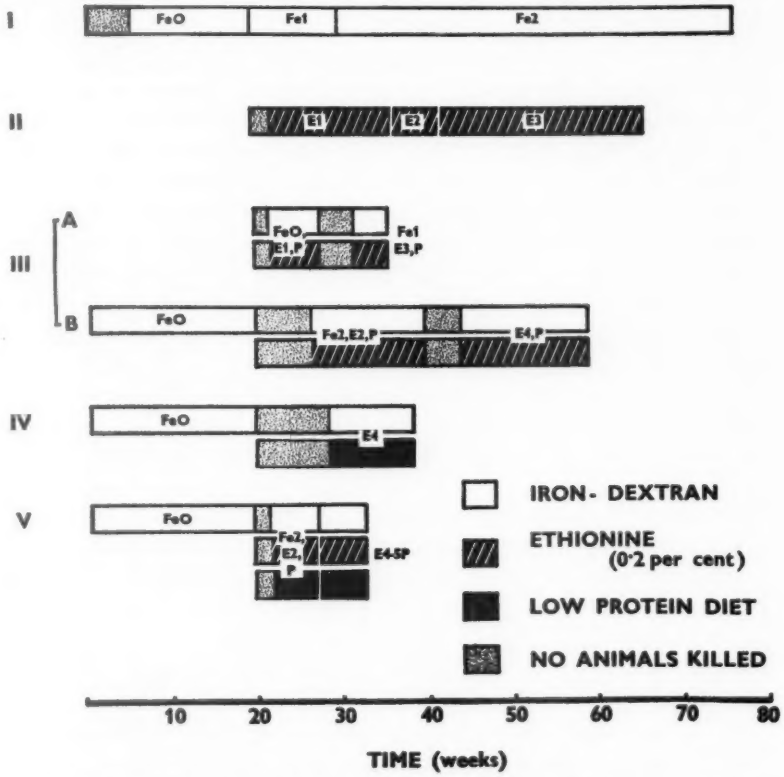
ship between the times of onset of some of the principal pathologic changes. Once again, because of the close similarity in groups IIIB and IIIC, and groups VA and VB, only the results for the two comparable groups, IIIB and VA are shown.

Pancreatic atrophy was induced by ethionine but was absent in iron-




TEXT-FIGURE 8. Time changes in the pattern of iron and ceroid distribution, and the development of cirrhosis in iron-laden rats placed on a low protein and ethionine diet after an interval of 20 weeks.

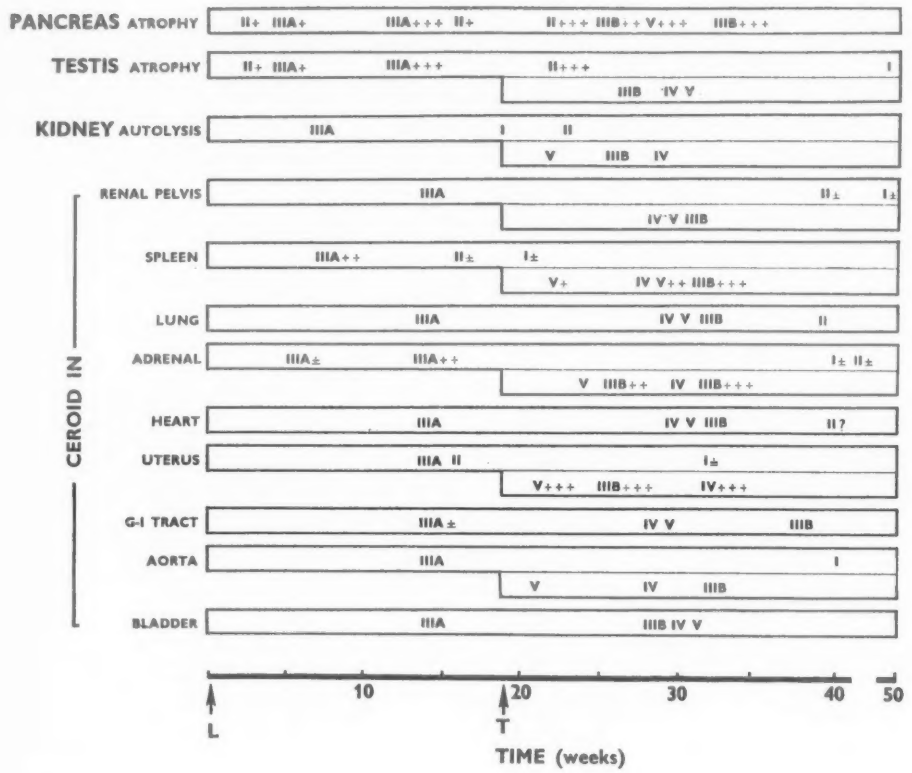
loaded rats on the basal or the low protein diets. Concurrent iron loading and ethionine (group IIIA) greatly hastened the speed with which atrophy developed, but this was not the case when a substantial interval (L-T) separated the start of the two treatments. The absence of pancreatic damage in the low protein group IV is not in keeping with the




TEXT-FIGURE 9. Summary of liver changes in the different experimental groups (groups IIIB and VA have been amalgamated with groups IIIC and VB, respectively).

statement by Kaufman, Klavins and Kinney[7] that "diets low in protein with or without high fat content produce pancreatic damage." In fact, our diet was similar to that used by György and Goldblatt,[8] who reported no abnormality in the pancreas. The lard content, 20 per cent, was much below that used by Kaufman and co-workers (60 per cent).

Testicular atrophy was infrequent in iron-loaded rats and was not seen at all in the males of group I (the time of 50 weeks shown in Text-figure 10 is based on previous results). With ethionine, the effect was manifest within 4 weeks, with complete atrophy at 23 weeks. Once again the




TEXT-FIGURE 10. Times of appearance of changes in other organs. L = start of iron loading (groups I and III to V) or of ethionine diet (groups II and IIIA). T = start of ethionine and/or low protein diet (groups IIIB, IV and VA). In the case of the pancreas, lung, etc., where a change is not observed in iron-laden rats of group I, the lower half of the time scale has been omitted since the interval between L and T is unlikely to be of importance.

change was accelerated in group IIIA. This striking result was matched by the observations on renal autolysis (the alteration seen when the excised rat kidney was kept at room temperature for 3 hours—a phenomenon hitherto considered characteristic of vitamin E deficiency in the rat). We have previously reported[4] that on diet 41 this alteration is first seen in the iron-loaded rat at about 9 weeks. It came as a surprise to find that ethionine was also capable of inducing this effect. Even more unexpectedly, in group IIIA renal autolysis was observed after only 7 weeks; on a vitamin E deficient diet the phenomenon was fully developed only after about 40 weeks.[9]

The formation of ceroid in various organs parallels its incidence in the liver. Without exception the ethionine diet with concurrent iron loading greatly hastened the appearance of ceroid. In some organs—lung, heart, gastrointestinal tract and bladder—no ceroid at all could be seen with iron loading alone. In others the scanty ceroid obtained with iron or ethionine alone was greatly augmented and appeared earlier as a consequence of the combined treatment. Where 12 or more weeks elapsed between the end of iron loading and the start of ethionine, the synergism was less obvious. Low protein diet in several instances displayed the same effect, and the combined low protein-ethionine diet lacking added vitamin E gave rise to ceroid in iron-treated animals within a few weeks.

The only distinct effect of sex was seen in group II, on ethionine diet only, where there was more ceroid in the kidney, spleen, lung and adrenal of females. On the whole, gonadectomy had little influence on the results.

## Discussion

### *Liver Iron and Ceroid*

A remarkable feature of the iron-loaded rat liver was the apparent migration of the iron-loaded Kupffer cells to form aggregates around the portal tracts and central veins and the late appearance of iron in the parenchyma. The first effect of ethionine was to enhance the deposition of parenchymal iron greatly and to accelerate considerably the migration of Kupffer cells especially when cirrhosis was developing. The aggregates were also much larger than those seen in purely iron-laden animals. In the cirrhotic livers there appeared to be two generations of cells in each aggregation of siderophages: (a) central cells heavy with iron and containing only a little ceroid, probably resulting from the injected iron; and (b) external to these, cells containing less iron but much more ceroid, perhaps stemming from destroyed parenchymal cells.

Before cirrhosis developed, all the parenchymal cells of iron-loaded

animals given ethionine contained a little ceroid and were heavily laden with iron. In the early development of cirrhosis seen in group IIIA, many parenchymal cells contained no iron; this was more apparent in the later stages of cirrhosis in group IIIB. We presume that these iron-free cells were newly formed. In the even later stages of cirrhosis in group V, the new parenchymal cells were taking up iron and had again begun to develop ceroid. It is evident from these changes in parenchymal iron and ceroid that a completely new generation of parenchymal cells developed in the cirrhotic livers of rats given ethionine.

Parenchymal ceroid was not more abundant in fatty cells, though Kupffer cells in areas of fatty parenchyma contained more ceroid than Kupffer cells in nonfatty parenchyma. However, it is known that as cirrhosis develops the fat tends to disappear,[5] and this may well be the case in the present groups. Taking the experiments as a whole, the development of ceroid was a clear example of synergy between the factors which provided conditions favorable for its formation; namely, (a) excess of intracellular fat, especially unsaturated fat; (b) excess of intracellular iron; (c) inadequacy or deficiency of tissue antioxidants such as vitamin E; (d) parenchymal, probably mitochondrial, damage.

### *The Relationship of Ethionine and Low Protein Diets to Vitamin E*

Our results show that ethionine given alone over a long period could produce effects suggestive of vitamin E deficiency. These included depigmentation of the incisors, enhanced renal antolysis, and the ceroid-laden "brown" uterus. Such changes were never seen in control animals. To what extent the testicular atrophy due to ethionine is preventable by vitamin E remains to be ascertained.

Much work points to a close relationship between vitamin E and the sulfur amino-acids (summarized by Beckmann[10]). More generally, the protective action of tocopherol in liver damage caused by various low protein diets has been fully established. The metabolic relationship between methionine and vitamin E has been attributed to a synergistic protection of labile sulfhydryl groups such as those of coenzyme A[11] or of glutathione.[12] It has been claimed that synergistic action of methionine will more than triple the antioxidant activity of α-tocopherol in stabilizing animal fats.[13] The "pro-oxidant" property of iron and the antagonism of methionine by ethionine would thus lead one to expect that iron and ethionine would each reinforce the anti-tocopherol effect of the other. Our evidence that such is the case indicates that the methionine-vitamin E relationship must rest on some basis other than antioxidant activity: there is no reason why ethionine should not be as good a "co-antioxidant" as methionine.

Ethionine reduces the methionine supply of methyl groups required for choline synthesis.[14] The striking effect on hepatic microsomal enzyme systems in the ethionine treated rat[15] and particularly the inhibition of intracellular protein synthesis and degradation[16,17] readily explain why protein depletion enhances ethionine-induced pancreatic damage.[18] In view of the intimate relationship existing between protein and vitamin E,[19,20] it is apparent that ethionine in a low protein diet, with unsaturated fat and no added vitamin E, may be expected to provide powerful support for the action of heavy stores of iron.

### *The Hepatotoxicity of Excessive Amounts of Stored Iron*

The clinical improvement brought about by repeated venesection in patients with hemochromatosis has made it clear that excessive amounts of stored iron exercise some sort of toxic action. Throughout our studies we have been impressed by the dynamic character of stored iron. This fact must be taken in conjunction with the difficulty of detecting iron histochemically until appreciable amounts of intracellular iron are present. Together, they render superfluous any discussion of parenchymal and mesenchymal iron as separate and distinct entities. This is an important point in considering the histogenesis of hepatic fibrosis which has been thought to result from stored iron. A primary reaction of reticuloendothelial cells, with a large increase in reticulin and ultimately of collagen, is considered by Popper and his colleagues[21] to be the outcome of extracellular iron storage in portal tracts. But as Walters and Waterlow[22] have pointed out, portal reticulosis alone is an extremely common reaction. The progression to frank fibrosis requires the intervention of a second factor, which the Gillmans[23] have postulated to be a persistent depression of oxidation. We have failed to find any depression of respiration in slices of iron-loaded rat liver and feel that the second factor is unconnected with the iron *per se*. The "second factor" may be endogenous damage to the liver associated with the cause of the siderosis —as in severe hemolytic anemia. Or it may arise from an exogenous dietary or toxic factor. Our experiments illustrate vividly the interaction of hepatic siderosis with "second factors" of this kind.

The apparently normal respiration of iron-loaded rat liver constitutes a phenomenon which, for lack of a better term, we propose to call "equilibrated swamping" of the body with iron. There can be but few instances—in fact we know of no other—in which a major bodily constituent can be retained in amounts as great as 300 times or more the normal total body content of that material without lethal effects. Even more surprising is the ability of the animal to live its normal span, apparently in good health. We have shown, however, by our earlier work

on the development of certain features suggestive of vitamin E deficiency and by our present experiments, that the iron-loaded rat exists in a condition of "equilibrated swamping." While under optimal circumstances this state is compatible with health and prolonged survival, it renders the animal particularly susceptible to nutritional, toxic and other metabolic hazards. This is but one more instance of the general rule that the metabolic state of an organism determines, in part at least, the manner in which it reacts to a defined stimulus.

Nothing can be said at present concerning the precise nature of the metabolic state of the iron-loaded liver. Bound ferric iron may be expected to display nonspecific activity in direct oxidations of sulfhydryl groups, tocopherol or adrenalin, just as it is claimed to do with toxins.[24] It might well act indirectly by bringing about peroxidation of unsaturated fatty acids and thus lead to inhibition of oxidative mitochondrial enzymes[25] possibly by slow reaction with sulfhydryl groups. The various possibilities are certainly not mutually exclusive but may be coupled; for instance, sulfhydryl or other reduction of ferric to ferrous iron may lead to release of the latter for other functions, such as its role in specific enzyme systems, especially DPN-cytochrome *c* reductase, of which α-tocopherol has now been found to be a cofactor.

### *The Final Common Pathway*

Matet, Matet and Friedenson[26] considered the protective action of tocopherol against liver necrosis to be nonspecific. Himsworth[27] dismissed the presence of ceroid as "an incidental result of the diets used." The possibility exists that these are all aspects of one and the same phenomenon, so that ceroid may be regarded as a pointer to one final common pathway taken by disturbances of liver metabolism with a wide diversity of origin. It is conceivable that the normal antioxidant protection of the unsaturated lipids which are bound up with the mitochondrion is adversely affected in the course of the mitochondrial changes through which the action of hepatotoxic agents is mediated.[28,29] As previously mentioned, damage of this kind may involve the accumulation of iron. Given sufficient time, this would prepare the ground for ceroid synthesis. When the affected cell comes to be replaced, the ceroid is carried by macrophages to lodge in centrilobular or periportal mesenchymal tissue.

It must be remembered that histochemical methods are insensitive in demonstrating intracellular iron and fail to reveal iron in a cell with increased ferritin content. Equally, when ceroid is seen, it represents a stage of polymerization whose earlier forms are difficult to distinguish with certainty. For this reason there is nothing unusual in finding hepatic hemosiderin without demonstrable ceroid or the reverse. In this way both

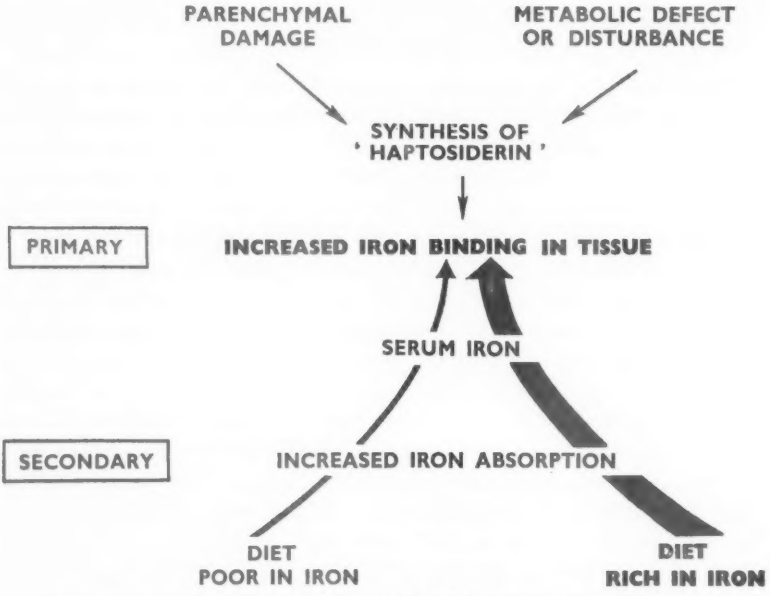
have come to be regarded as incidental findings and their true importance as twin indicators of potential or actual cellular damage overlooked. In the present experiments the stage has been set by providing the substrate (unsaturated fat) and the catalyst (iron) while the protective agent (tocopherol) has been eliminated, partly by omission from the diet and partly by the action of the stored iron. But these players alone do not constitute a cast, as can be seen by the vast increase in ceroid, as well as its rapidity of formation, when a nutritional or toxic factor is added.

### *Relevance of These Observations to Human Siderosis and the Problem of Hemochromatosis*

Much of the difficulty and controversy which has arisen in the field of iron storage syndromes has its origin in the single-minded concentration on excessive intestinal absorption of iron. It is much more profitable to consider the question as primarily one of excessive iron-binding in the liver, from which the increased intestinal absorption follows secondarily. Even a cursory survey reveals that a wide variety of conditions not involving hemolysis or marrow hypoplasia can result in hepatic siderosis. By and large, such conditions have one factor in common: actual or potential liver damage, the latter taking the form of disordered metabolism of the hepatic cell. The suggestion is now put forward that such liver damage involves the synthesis of excessive amounts of an iron-binding material for which we suggest the name "haptosiderin." "Haptosiderin" is in all probability akin to the organic matrix of hemosiderin, recently shown by Bielig and Wöhler [30] to contain a deoxyribonucleotide. Or it may be similar to the copper-binding polypeptides isolated by Uzman, Iber, Chalmers and Knowlton [31] from the liver in Wilson's disease. In any case it is likely to be capable of binding other heavy metals which in turn are displaced by iron.[32] Thus, in rats poisoned chronically with nickel or cobalt,[33] it is hemosiderin which accumulates in the liver, while in human hepatic iron accumulation there is also excessive storage of lead, molybdenum and copper.[34]

A possible relationship of "haptosiderin" synthesis in excessive amounts and siderosis of the liver is illustrated in Text-figure 11. By this means, for example, the apparently conflicting views with regard to the etiology of Bantu siderosis are readily reconciled: Gillman and Gillman [23] point to disordered metabolism of the hepatic cell; Walker and Arvidsson [35] draw attention to the heavy level of dietary iron. Both are partially correct. The amount of iron present in the liver is far too great to have arisen solely from disrupted mitochondria as the Gillmans assert, unless these mitochondria were loaded with iron in the first place [36]; on the other hand, the dietary iron alone cannot adequately account for the

enormously enhanced absorption from the intestine. If, in fact, hepatic derangement as a primary influence is responsible for increased iron uptake, all the known facts fall into place. Where either the hepatic disorder or the excess of dietary iron is lacking, siderosis is not seen—for instance, elsewhere in Africa or among other poorly nourished peoples. Similarly with experimental observations such as siderosis in ethionine, carbon tetrachloride and other intoxications. Kinney and co-workers[3] have sought to attribute the ethionine effect to pancreatic damage, thus




TEXT-FIGURE 11. Suggested role of "haptosiderin" in iron storage syndromes.

bringing it into line with the results of Taylor, Stiven and Reid[37] on hepatic siderosis after pancreatic duct ligation in the cat. On a low protein, 60 per cent lard diet, Kaufman and his colleagues[7] induced pancreatic damage as well as hepatic siderosis. There is ample evidence[38] to indicate that pancreatic damage is not uncommonly associated with hepatic derangement, which would thus adequately account for the observed siderosis.

Of the factors which may be concerned in Bantu siderosis, a low protein diet suggests itself. In idiopathic hemochromatosis, "haptosiderin" in excessive amounts might be attributable to some inborn error of metabolism which has far-reaching consequences throughout the body. "Haptosiderin" may also arise in chronic refractory anemias which

are treated with multiple transfusions. In this way one could account for the fact that occasionally far more iron is found in the liver than can be accounted for by the transfusions. Moreover, the degree of terminal liver damage is often unrelated to the amount of iron present.

## Summary

A gross degree of iron overload has been induced in rats. The siderotic but otherwise apparently normal liver displays an enhanced susceptibility to the action of dietary ethionine, or of a diet poor in protein and vitamin E, or more especially to the two factors acting together. Under this influence, hepatic cirrhosis, which does not supervene in the iron-loaded rat even after two years, makes its appearance within a few weeks, side by side with the massive aggregation of siderophages in the interlobular septums.

In other organs, lesions, some associated with lack of vitamin E, are correspondingly accelerated. The most striking of all these instances of synergy is the extensive development of ceroid throughout the body.

It is suggested that in the presence of tissue siderosis of such degree there exists a state of "equilibrated swamping" with regard to the iron, the equilibrium being precarious and, in the case of the liver, vulnerable to a host of adverse circumstances. An attempt is made to correlate the various human iron storage syndromes through a common mechanism of origin or development.

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[*Illustrations follow*]

## Legends for Figures

Figures 1 to 3 represent the livers of rats sacrificed at varying times after the start of injections totaling 1,650 mg. of iron per kg. of body weight.

Fig. 1. After 9 weeks. Iron is confined to the Kupffer cells, many of which are enlarged. Prussian blue stain, neutral red counterstain. × 150.

Fig. 2. After 24 weeks. Small aggregations of Kupffer cells in portal tracts, with a little iron in the periportal parenchyma. Prussian blue stain, neutral red counterstain. × 150.

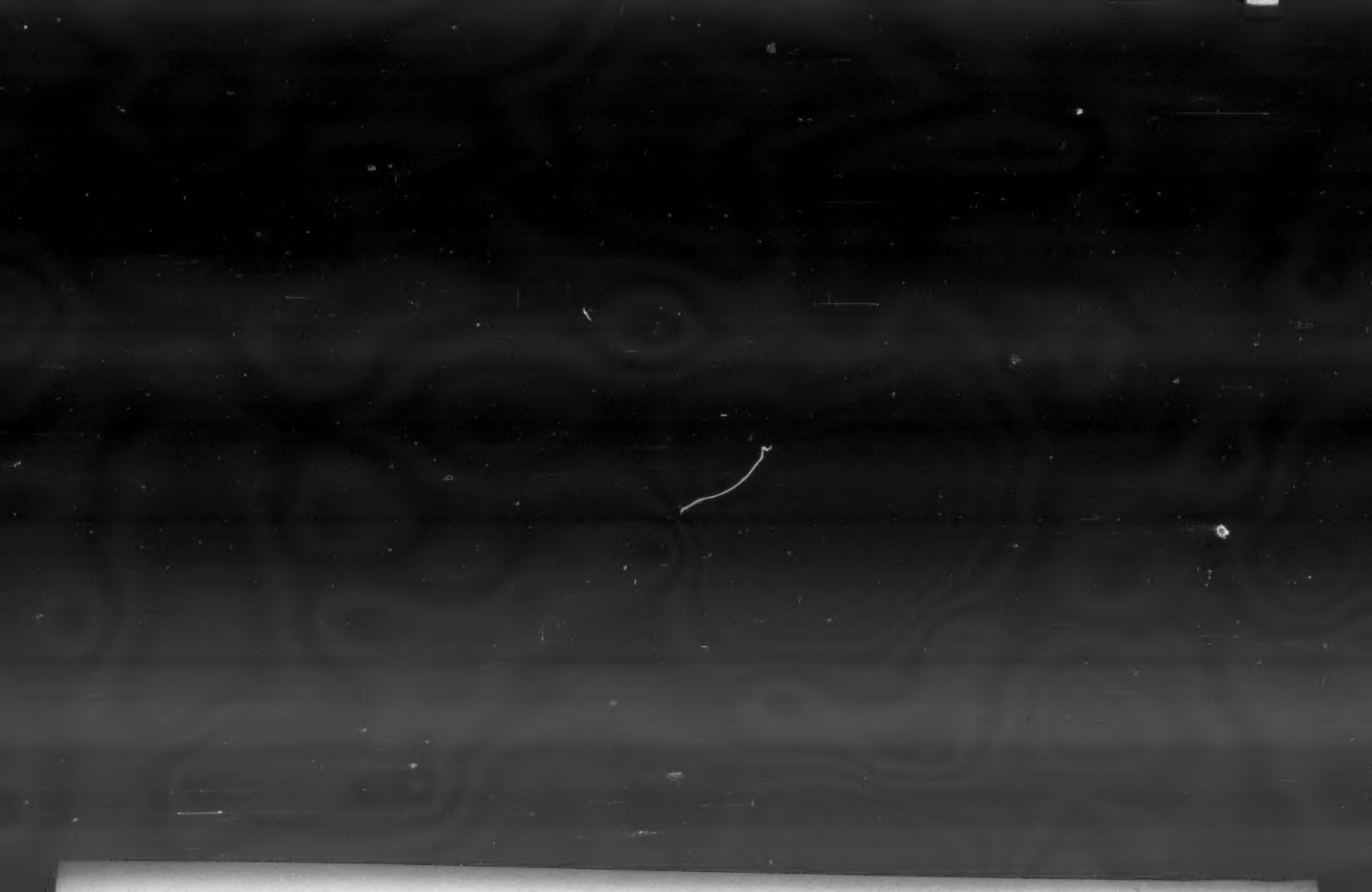
Fig. 3. After 76 weeks. Larger periportal and centrilobular siderophage accumulations with disappearance of iron from sinusoidal Kupffer cells and moderate amounts of iron in the periportal parenchyma. Prussian blue stain, neutral red counterstain. × 55.

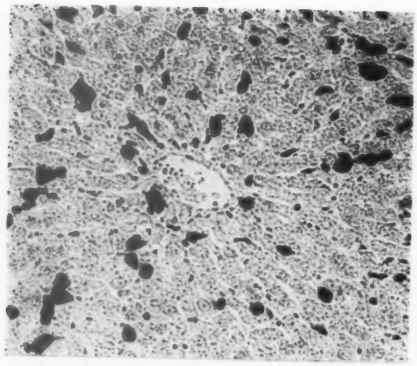
Figures 4 to 6 represent the livers of rats sacrificed at varying times after commencement of the ethionine diet.

Fig. 4. After 15 weeks. Centrilobular accumulation of iron-containing macrophages. Prussian blue stain, neutral red counterstain. × 200.

Fig. 5. After 22 weeks. Ceroid pigment in large macrophages in the periportal stroma and in a few parenchymal cells. Ziehl-Neelsen stain. × 510.

Fig. 6. After 39 weeks. Fully developed cirrhosis with small aggregates of siderophages in the fibrous septums. Prussian blue stain, neutral red counterstain. × 55.





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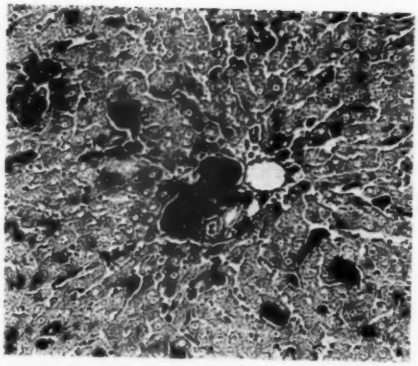

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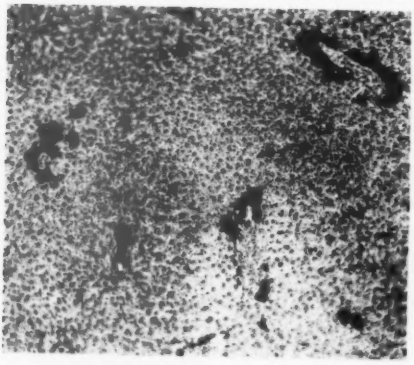

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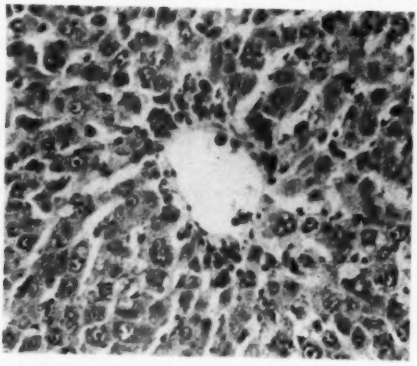

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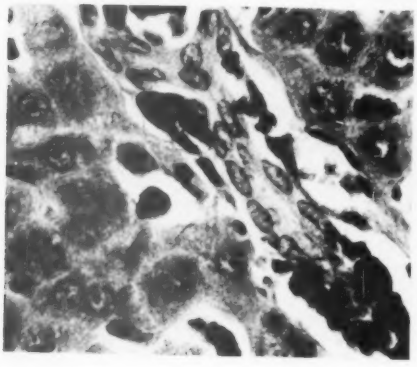

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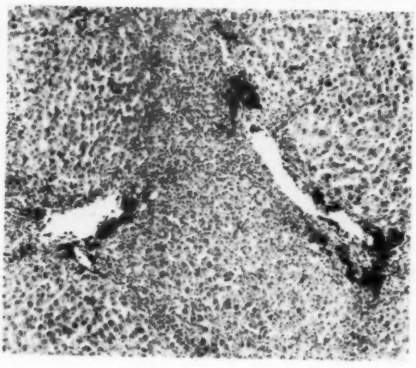

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Figures 7 and 8 represent livers of rats sacrificed after commencement of concurrent treatment with iron and ethionine.

FIG. 7. After 5 weeks. Uniformly heavy iron deposition in Kupffer cells and throughout the parenchyma. Prussian blue stain, neutral red counterstain. × 150.

FIG. 8. After 14 weeks. Early monolobular cirrhosis with very heavy iron deposits in the fibrous septums and, irregularly, in the sinusoidal Kupffer cells and parenchyma. Prussian blue stain, neutral red counterstain. × 55.

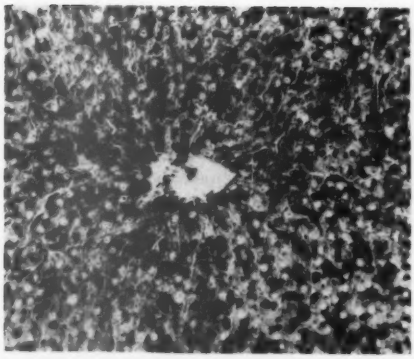
Figures 9 and 10 are sections from the livers of rats treated with iron followed by ethionine after an interval of 20 weeks.

FIG. 9. After a total of 32 weeks. Centrilobular and periportal aggregates of siderophages, with heavy diffuse parenchymal iron. Prussian blue stain, neutral red counterstain. × 55.

FIG. 10. After a total of 56 weeks. Advanced cirrhosis, with heavy iron in the fibrous septums. Prussian blue stain, neutral red counterstain. × 55.

FIG. 11. Liver of a rat on a low protein diet for 12 weeks. The diet was commenced after an interval of 20 weeks following iron loading. A fine cirrhotic pattern with heavy stromal iron and complete absence of iron from parenchymal cells. Prussian blue stain, neutral red counterstain. × 55.

FIG. 12. Liver of a rat after a total of 32 weeks; a low protein and ethionine diet had been fed after an interval of 20 weeks following iron loading. Severe cirrhosis with heavy stromal iron and iron in new parenchymal and Kupffer cells. Prussian blue stain, neutral red counterstain. × 55.



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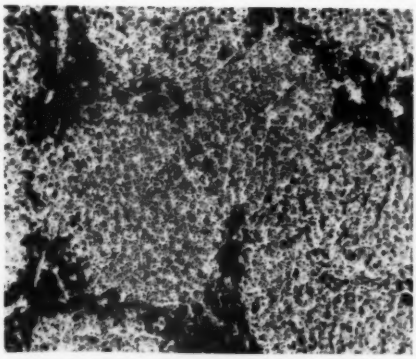

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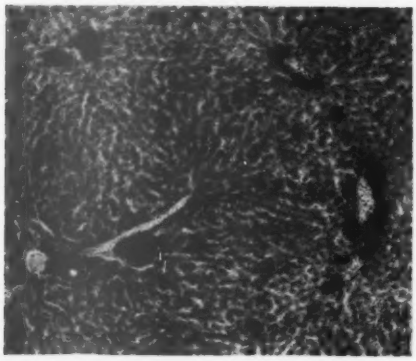

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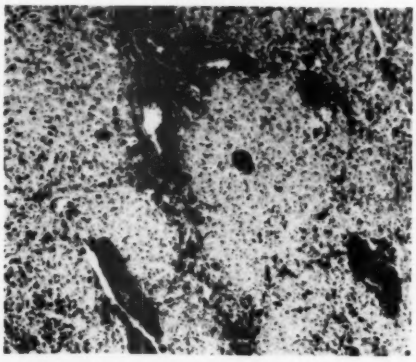

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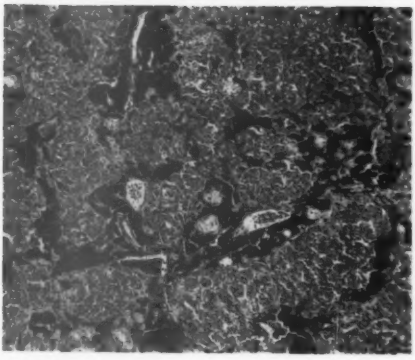

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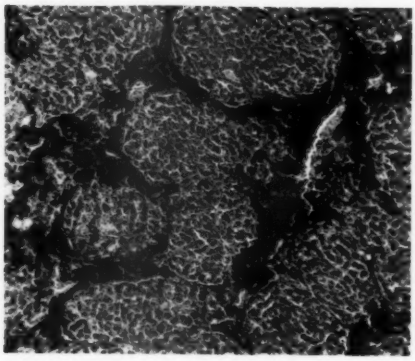

12

# THE PATHOLOGY OF NEONATAL HEPATITIS

B. Ruebner, M.D.*

*From the Department of Pathology, Dalhousie University, and the Pathology Institute of Nova Scotia, Halifax, N.S., Canada*

The clinical pattern of obstructive jaundice is not infrequently seen in infancy and in the neonatal period. Erythroblastosis, syphilis, cystic fibrosis of the pancreas and galactosemia are some of the less common conditions which may produce it.[1] These disorders are usually recognized by serologic or biochemical means. About 85 per cent of jaundiced infants, however, suffer from either congenital obliteration of the extrahepatic bile ducts or from a parenchymal liver disease with a normally patent biliary tree.[2] The latter condition is of uncertain etiology, and many names have therefore been applied to it. The most commonly used is neonatal hepatitis.[2]

This investigation describes the pathologic alterations in 4 cases of neonatal hepatitis with particular emphasis on the differentiation of this condition from extrahepatic biliary atresia.[3] In 2 of the cases of neonatal hepatitis, apart from excluding biliary obstruction, injection of India ink provided an opportunity to observe the intrahepatic biliary system.

## Material and Methods

Three babies suffering from neonatal hepatitis were examined at necropsy (cases 1, 2 and 4). Their ages at death ranged from 6 weeks to 3 months. In 2 of these infants (cases 1 and 2), the common hepatic duct was injected with approximately 5 ml. of a ⅓ dilution of India ink in distilled water. A liver biopsy specimen was obtained at laparotomy from another baby aged 2½ months (case 3).

Surgical biopsy specimens were received from 3 infants with biliary atresia between the ages of 3 and 18 months (cases 5, 6 and 8). Two specimens were obtained from one of these (case 5). A necropsy was performed on another patient aged 15 months (case 7).

Paraffin sections were stained with hematoxylin and eosin, by van Gieson's method, and for reticulin.[4] The Prussian blue reaction for ferric iron and the periodic acid-Schiff reaction with and without diastase digestion were also performed. Frozen sections were stained by Sudan IV for fat in cases where sufficient material was available.

* Present address: Department of Pathology, Johns Hopkins Hospital, Baltimore 5, Md.

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## Results

### *Clinical Observations*

The infants with neonatal hepatitis failed to thrive almost from birth. Three (cases 2, 3 and 4) were noted to be jaundiced from about the second week of life. Case 1 did not become jaundiced until the fourth week. Cases 1, 2 and 4 died from liver failure with ascites before the age of 3 months. Case 3, however, is now improving and appears to have a good chance of survival.

The babies with biliary atresia (cases 5, 6, 7 and 8) were jaundiced from birth. They gained some weight during the early months of infancy and, apart from their jaundice, were clinically fairly well. All survived at least 6 months. Later their condition deteriorated, and 2 are known to have died with ascites (cases 7 and 8). A necropsy was performed on only one of these (case 7).

The clinical differences between the two groups were not sufficiently marked to make possible a confident clinical distinction. Biochemical tests also gave little help in differential diagnosis. In all the babies, Rh incompatibility between mother and child was excluded. Serum tests for syphilis were negative in all cases. Both groups of babies were mildly anemic. Two cases of neonatal hepatitis (cases 2 and 4) had white blood cell counts of 22,000 and 48,000 per cmm. There was mild anemia, and the peripheral blood contained a few nucleated red blood cells.

### *Gross Observations*

The livers and spleens obtained at necropsy in the 3 patients with neonatal hepatitis were all enlarged. In cases 1 and 2 they were only slightly heavier than normal, but in case 4 the liver weighed 800 gm. (more than 4 times normal). All the livers were intensely green. The surface of 2 liver specimens (cases 2 and 4) was finely granular. The other (case 1) had a smooth surface with a scar about 2 cm. long in the right lobe. The patency of the biliary tract was proved by dissection in cases 1, 2 and 4, which were observed at necropsy. Injection of India ink into the common hepatic duct in cases 1 and 2 resulted in uniformly distributed filling of the intrahepatic bile ducts. In case 3, a cholangiogram at laparotomy showed a normal intra- and extrahepatic biliary tree.

Only one of the cases with biliary atresia was examined at necropsy (case 7). The liver weighed 400 gm. (normal, 300 gm.) and was green and finely nodular. There was atresia of the common bile duct 3 cm. below the junction of the cystic and common hepatic ducts. Above this level there was marked dilatation of the bile passages. The spleen weighed 190 gm. (normal 30 gm.). There were several simple cysts in the pan-

creas but no fibrosis. Atresia of the extrahepatic bile ducts, not correctable by surgery, was found at laparotomy in the other 3 patients.

### *Histologic Observations*

The histologic abnormalities of the liver in each of the 4 cases of neonatal hepatitis are enumerated in Table I where their severity is graded from ± to +++. Moderate numbers of giant parenchymal cells containing up to 30 nuclei were seen in 2 cases. In the other 2 cases only a few scattered giant cells could be found. These cells contained considerably more glycogen than most other hepatic cells. Frequently they were also heavily laden with hemosiderin, and sometimes they showed degenerative changes. A duct-like arrangement of the liver cords, often with a bile thrombus in the center, was very frequent in

TABLE I

HISTOLOGIC APPEARANCE OF LIVER IN 4 CASES OF NEONATAL HEPATITIS

| | Case 1 | Case 2 | Case 3 | Case 4 |
|---|---|---|---|---|
| Age: | 1½ mos. | 1½ mos. | 2½ mos. | 3 mos. |
| Giant cells | + | + | ± | ± |
| Duct-like liver cells | ++ | ± | ± | ± |
| Liver cell degeneration | + | + | + | + |
| Liver cell necrosis | + | + | + | + |
| Intralobular inflammation | ++ | + | + | + |
| Interlobular inflammation | + | ++ | ++ | + |
| Bile duct proliferation | − | ++ | ± | + |
| Ductular hyperplasia | + | + | − | + |
| Intracellular bile stasis | + | + | + | ± |
| Bile thrombi in canaliculi | + | + | + | ± |
| Interlobular bile thrombi | − | + | − | − |
| Intralobular fibrosis | + | + | ± | + |
| Perilobular fibrosis | ± | ++ | ± | + |
| Foci of hematopoiesis | − | ± | + | + |
| Hemosiderin in liver cells | ++ | + | ± | ± |
| Regenerative nodules | − | − | − | − |
| Fatty alteration | + | ± | | + |

case 1. In the other specimens this feature was not marked. Degenerative changes were often seen in parenchymal elements. Hydropic degeneration of the cytoplasm was common in all cases. Eosinophilic degeneration was less evident. Enlargement appeared to be the first nuclear alteration. Karyorrhexis, karyolysis and nuclear pyknosis often followed. Acute and chronic inflammatory cells were seen not only in the portal triad area but were also scattered diffusely through the lobules. All except the case with the mildest lesion (case 3) showed an increase in elongated fibroblast-like cells between liver cords. These cells often formed lumens which were frequently filled by injection of India ink into the common hepatic duct (Fig. 1). The lumens were therefore thought to be hyperplastic

biliary ductules (canals of Hering). Proliferation of bile ducts in the portal areas was marked only in case 2 (Fig. 2). Bile stasis was seen in all instances, predominantly with intracellular and canalicular location (Fig. 3). Interlobular bile stasis was seen only in case 2; there was also marked proliferation of the portal bile ducts in this instance (Fig. 4). In

TABLE II

HISTOLOGIC APPEARANCE OF LIVER IN 5 SPECIMENS FROM 4 CASES OF EXTRAHEPATIC BILIARY ATRESIA

| | Case 5 | | | | |
|---|---|---|---|---|---|
| | (spec. I) | (spec. II) | Case 6 | Case 7 | Case 8 |
| Age: | 3 mos. | 7½ mos. | 3 mos. | 15 mos. | 18 mos. |
| Giant cells | ++ | − | − | − | − |
| Duct-like liver cells | − | ± | ± | − | − |
| Liver cell degeneration | ++ | + | + | ± | ± |
| Liver cell necrosis | + | ± | ± | ± | + |
| Intralobular inflammation | ± | ± | ± | ± | + |
| Interlobular inflammation | ++ | + | + | + | + |
| Ductular hyperplasia | − | − | − | − | − |
| Increase of bile ducts | ± | ++ | ++ | + | + |
| Intracellular bile stasis | + | + | + | ± | + |
| Bile thrombi in canaliculi | + | + | + | + | + |
| Interlobular bile thrombi | ± | − | + | + | + |
| Intralobular fibrosis | − | − | − | ± | − |
| Perilobular fibrosis | ± | ++ | ++ | +++ | +++ |
| Foci of hematopoiesis | − | − | − | − | − |
| Hemosiderin in liver cells | ++ | − | + | + | − |
| Regenerative nodules | − | − | − | + | + |
| Fatty alteration | | | | ± | |

both cases where the common hepatic duct was injected, India ink particles were frequently seen immediately adjacent to bile thrombi (Figs. 3 and 4). The pattern of fibrosis was characteristically intralobular, surrounding cords of liver cells rather than entire lobules (Fig. 1). Perilobular fibrosis was marked only in the one specimen with marked portal bile duct proliferation (Fig. 2). Foci of hematopoiesis were seen in the liver in 3 of 4 cases. In cases 2 and 4 there was, in addition, marked hematopoietic activity in the spleen and lymph nodes. Hemosiderin granules were present in the hepatic cells of all subjects in varying quantities. More rarely they were seen in Kupffer cells. The pancreas was histologically normal in the 3 necropsied cases.

For comparison, the histologic features in 5 liver specimens from 4 cases of extrahepatic biliary atresia are summarized in Table II. Fibrosis and bile duct hyperplasia were characteristically perilobular (Figs. 5 and 6). Giant cell formation, degenerative changes and a duct-like arrangement of liver cells were frequent (Figs. 7 to 9). Necrosis and inflammation tended to be focal (Fig. 10). Hematopoiesis was not seen.

## Discussion

In our cases, as in previous investigations,[2] it was clinically difficult to distinguish neonatal hepatitis from biliary atresia. Pathologically, atresia of the intrahepatic bile ducts could be excluded in all cases because bile ducts were present in portal tracts.[6] Typical neonatal hepatitis (cases 1 and 4) was easily distinguished from the well established examples of extrahepatic biliary atresia (cases 6, 7 and 8). Difficulties were encountered, however, in two cases of neonatal hepatitis, one of which was mild (case 3) and the other atypical (case 2), and also in an early case of biliary atresia (case 5, specimen I). The histologic criteria for neonatal hepatitis[2,3] and those for extrahepatic biliary atresia,[6] therefore, require discussion.

Prominent hepatic giant cells have been considered suggestive of neonatal viral hepatitis.[7] Craig and Landing,[2] however, found giant cells in a third of their patients with biliary atresia. In the present series, giant cells were more prominent in the earlier specimen from case 5 (Fig. 7), a patient with biliary atresia, than in any of the infants with neonatal hepatitis. They cannot, therefore, be of much help in distinguishing atresia from neonatal hepatitis. There are two views concerning the origin of the giant cells. It has been proposed that they may arise by dissolution of cell boundaries[2] or by proliferation of isolated liver cells.[3] There may be some truth in both these theories. In the present investigation the boundaries between liver cells with evidence of degeneration were often hardly perceptible, and it was difficult to decide whether the nuclei belonged to a cord of individual cells or were part of a multinucleated giant cell. Although mitotic figures were not seen in any giant cells, two histologic features did suggest that they were metabolically active. First, they contained a disproportionately large number of nuclei; second, there was a heavy deposit of glycogen.

A duct-like arrangement of liver cells was seen occasionally in biliary atresia (Fig. 8) but much more frequently in neonatal hepatitis. This feature is therefore suggestive of neonatal hepatitis.[3]

Hydropic alteration and eosinophilic degeneration of liver cells have been considered indicative of viral hepatitis.[8] These features were particularly prominent in case 5, specimen I, and case 6, both examples of biliary atresia (Fig. 9). Degeneration of liver cells, therefore, is not helpful in differential diagnosis.[6]

In both groups of patients in this investigation, neutrophils and chronic inflammatory cells were frequently seen adjacent to necrotic liver cells. In biliary atresia this infiltrate was focal (Fig. 10) whereas in neonatal hepatitis it was more diffuse.[2] A portal inflammatory reac-

tion, however, had no value in differential diagnosis, as it was encountered frequently in both conditions.

Some hyperplasia of the perilobular bile ducts was seen in all of the patients with biliary atresia. This was greater in the older patients (case 7) and barely perceptible in the first specimen from case 5, a baby aged 3 months (Fig. 5). In neonatal hepatitis, Craig and Landing[2] observed only minimal bile duct proliferation. Dible,[3] however, found bile duct hyperplasia in 7 of 8 cases. In our cases with well developed lesions the hyperplasia differed from that of biliary atresia in two respects. First, it affected the intralobular ductules (which were filled with India ink in the two cases where the common hepatic duct was injected) as well as the perilobular bile ducts (Fig. 1). Second, the hyperplastic bile ducts in neonatal hepatitis were more atypical than those in biliary atresia. The intralobular bile duct hyperplasia of neonatal hepatitis is similar to that produced in rats by prolonged intoxication with alpha-naphthyl-isothiocyanate.[9] One patient (case 2) showed marked perilobular bile duct proliferation and a duct-like arrangement of many groups of liver cells (Fig. 2). In both respects this resembles case 7 of Dible[3] and also other instances of cholangiolotic hepatitis[10] and infectious cirrhosis.[11] The only histologic difference between these lesions and those in the more usual examples of neonatal hepatitis is the perilobular bile duct proliferation. True infectious cirrhosis[11] has a patchy lesion; in our case 2, as in those of Dible[3] and Peace,[10] the alterations were diffuse, and no source of any primary infection was found. It seems more likely that these cases have a cause similar to that in the more common type of neonatal hepatitis with predominantly intralobular ductular proliferation. Specimens characterized by marked perilobular bile duct proliferation may well correspond to the "cholangiolitic" hepatitis of adults.[12]

The absence of interlobular thrombi in neonatal hepatitis has been stressed.[2] In our cases, interlobular bile stasis was more marked in biliary atresia but also occurred in neonatal hepatitis (Fig. 4). It was absent in one specimen from case 5, a patient with atresia, originally thought to have neonatal hepatitis.

The pattern of fibrosis in well developed cases of biliary atresia was entirely different from that in neonatal hepatitis. In atresia the fibrosis was almost entirely perilobular[8] (Fig. 6). In neonatal hepatitis the widening of the portal areas was mild even in advanced cases[2] (Fig. 1). Intralobular fibrosis, however, was widespread and surrounded isolated groups of liver cells[3] (Fig. 2). The distribution of the fibrosis in both biliary atresia and neonatal hepatitis was, therefore, similar to that of the bile duct hyperplasia in these conditions. In experimental liver injury also, a ductular cell reaction may produce cells closely resembling fibroblasts.[13]

Extramedullary hematopoiesis has often been reported in neonatal hepatitis,[2,8,10] but apparently never in biliary atresia. In this series it was present in 3 of 4 cases of neonatal hepatitis, but in none of those with biliary atresia. The presence of extramedullary hematopoiesis, therefore, seems to be suggestive of neonatal hepatitis if blood group incompatibility can be excluded. In this group of cases no bone marrow abnormality was found, and the basis for the occurrence of extramedullary hematopoiesis remains obscure.

Dible [3] stressed the heavy infiltration of liver cells with stainable iron in neonatal hepatitis. As there was no evidence of blood destruction, this observation was considered to be evidence of liver cell dysfunction. In the present investigation a strongly positive Prussian blue reaction was found, not only in the tissue from babies suffering from neonatal hepatitis but also in 3 of 4 cases with biliary atresia. The severity of this alteration ran parallel with other features of liver cell damage. Hemosiderosis of liver cells thus had no value in distinguishing the lesions of neonatal hepatitis from those of atresia.

When neonatal hepatitis was first described as an entity,[2,8,7] it was generally thought to be caused by a virus. This view was challenged by Smetana and Johnson [14] who believed it to be due to a failure of development of the intercellular bile canaliculi. Hsia, Boggs, Driscoll and Gellis [15] examined 120 patients from 39 families and found the incidence of this condition consistent with an autosomal recessive inheritance, but they could not exclude an extrauterine factor. Although infectious hepatitis in pregnancy generally does not affect the fetus,[16] a few cases have been reported when hepatitis in pregnant women was followed by hepatitis in the infant.[17,18] The mothers of several other infants with hepatitis showed evidence of liver damage possibly due to the virus of homologous serum jaundice.[19-22]

The observations in our two cases of neonatal hepatitis in which India ink was introduced into the common hepatic duct do not support the views of Smetana and Johnson.[14] In both instances ink particles could be seen in canaliculi immediately adjacent to bile thrombi (Figs. 3 and 4), thus proving the absence of any organic obstruction of the intrahepatic biliary system. It seems more likely that the bile stasis in these cases was due to hepatocellular disease, probably of viral origin. Certainly a few instances of neonatal hepatitis appear to be the result of cytomegalic inclusion disease.[23] The cause of the majority of cases is still uncertain, however. The histologic pattern of neonatal hepatitis differs from that of infectious hepatitis of adults in some respects. Until the virus or viruses of hepatitis can be demonstrated, uncertainty as to whether this variation is due to differences in etiologic agents or merely to the difference in the affected age groups must remain.

## Summary

The histologic features in 4 cases of neonatal hepatitis are described and compared with those in 4 cases of extrahepatic biliary atresia. In some instances the distinction between the two conditions proved difficult.

Parenchymal giant cells and a duct-like arrangement of liver cells were more often seen in neonatal hepatitis than in biliary atresia. This difference in frequency, however, was insufficient to be of help in diagnosis. Hydropic degeneration, eosinophilic necrosis, and hemosiderin deposition in liver cells were common in both conditions. In neonatal hepatitis there seemed to be proliferation of intralobular bile ductules. Perilobular bile duct proliferation and bile stasis were more frequent in biliary atresia but were prominent also in one case of neonatal hepatitis.

This variety of neonatal hepatitis is likened to the "cholangiolitic" type of hepatitis in adults. The distribution of the intralobular inflammatory cells differed in the two conditions. It was focal in biliary atresia but diffuse in neonatal hepatitis. The pattern of fibrosis in established cases of biliary atresia was almost entirely perilobular, while in well developed neonatal hepatitis intralobular fibrosis surrounded isolated groups of liver cells. Extramedullary hematopoiesis was marked in neonatal hepatitis but did not occur in biliary atresia.

India ink was injected into the common hepatic duct in two cases of neonatal hepatitis. The injected ink particles could be seen in canaliculi or bile ducts immediately adjacent to bile thrombi, thus proving the absence of any organic obstruction of the intrahepatic biliary system. It was concluded that bile stasis in these patients was due to a hepatocellular disease, probably of viral origin.


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I am most grateful to Prof. J. H. Dible, Dr. H. Popper and Dr. J. M. Craig for their opinions on some of these cases, and to Mr. C. S. Brindle for taking the photographs.

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[*Illustrations follow*]

## Legends for Figures

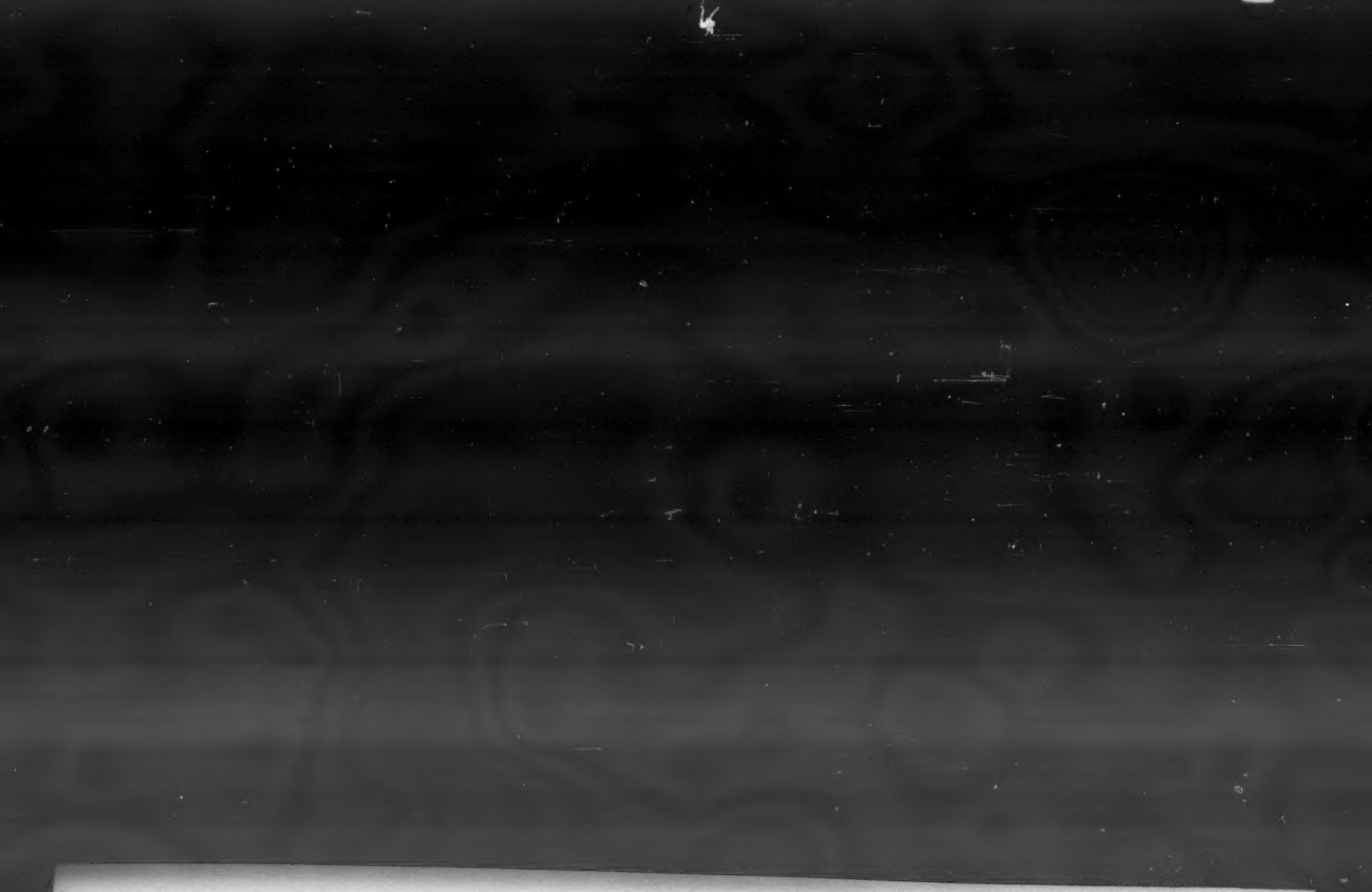
Except where indicated, illustrations were prepared from sections stained with hematoxylin and eosin.

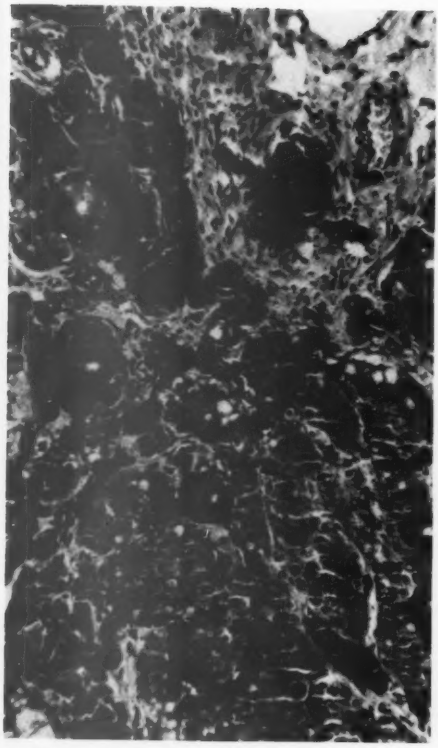
Fig. 1. Neonatal hepatitis (case 1). A portal bile duct is filled with India ink. This material also penetrates between cords of liver cells into what appear to be hyperplastic bile ductules (canals of Hering). Note intralobular fibrosis and inflammation. × 110.

Fig. 2. Neonatal hepatitis (case 2). This is the only neonatal hepatitis specimen showing marked proliferation of portal bile ducts. Some portal bile ducts contain India ink. Perilobular fibrosis and conspicuous intralobular fibrosis are also present. × 110.

Fig. 3. Neonatal hepatitis (case 1). At the center there is an intracanalicular bile thrombus. India ink particles are immediately adjacent to it. × 270.

Fig. 4. Neonatal hepatitis (case 2). There are two interlobular bile thrombi. Several of the hyperplastic portal bile ducts are filled with India ink. In the center, a bile thrombus is immediately adjacent to ink particles. × 270.





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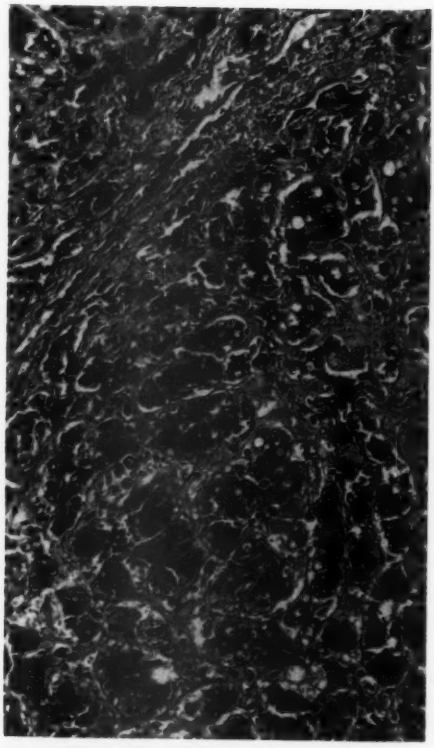

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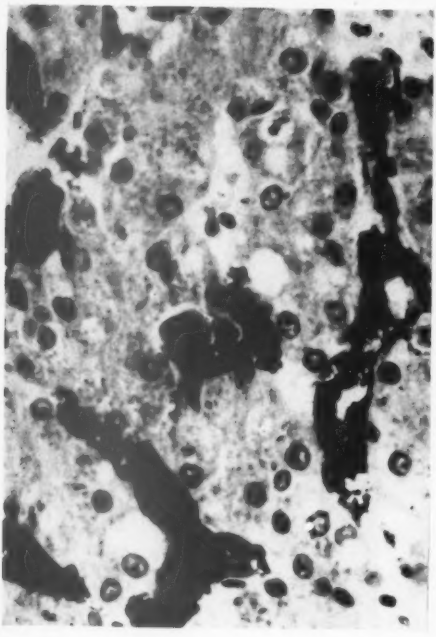

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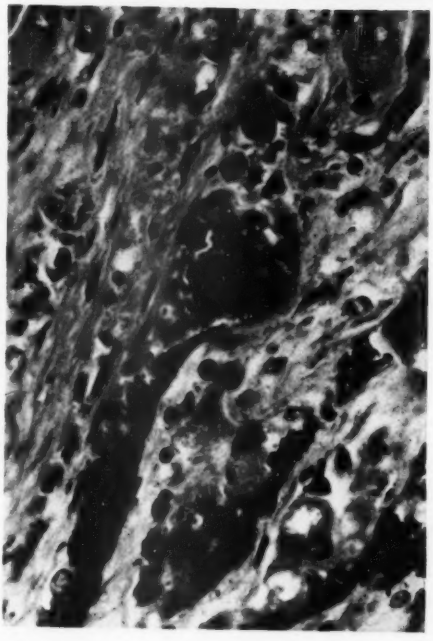

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FIG. 5. Biliary atresia (case 5, aged 3 months; specimen I). Most of the portal areas in this first specimen were normal. One of the few portal triads is shown which exhibits ductal hyperplasia and some increase in connective tissue. Silver impregnation for reticulin. × 110.

FIG. 6. Biliary atresia (case 8, aged 18 months). Compare this liver biopsy specimen with Figure 5. There is a marked increase in perilobular fibrosis and a decrease in the number of interlobular bile ducts. Note the absence of intralobular fibrosis as compared with Figure 2. At the lower right corner, there is a bile thrombus in an interlobular bile duct. × 110.

The remaining illustrations are from case 5, specimen I (biliary atresia).

FIG. 7. Parenchymal multinucleated liver giant cell. The surrounding cells show hydropic degeneration. × 270.

FIG. 8. The liver cord in the center is arranged in a duct-like pattern and surrounds a bile thrombus. × 270.

FIG. 9. In the center there is a binucleate liver cell undergoing eosinophilic degeneration. One nucleus is pyknotic. The surrounding cells show hydropic degeneration. × 270.

FIG. 10. In the center there is a focus of necrosis infiltrated by acute and chronic inflammatory cells. × 270.

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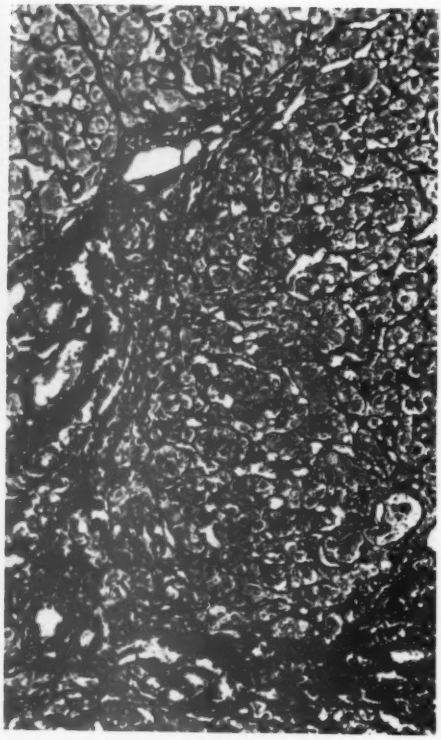

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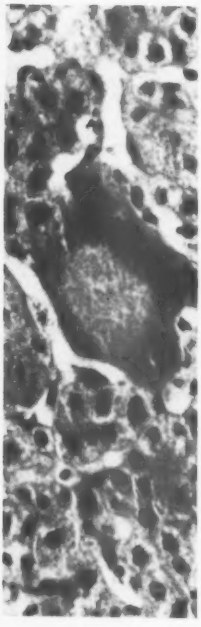

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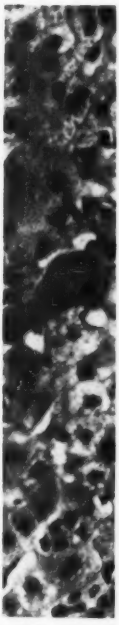

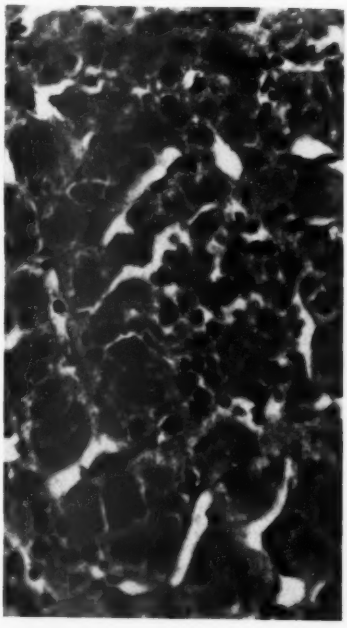

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# AGE CHANGES IN THE RENAL BASEMENT MEMBRANE IN RATS

C. T. Ashworth, M.D.; R. R. Erdmann, M.D., and N. J. Arnold, B.S.

*From the Department of Pathology, The University of Texas Southwestern Medical School, Dallas, Texas*

Basement membrane structures throughout the body have been the object of extensive observation and discussion by histologists and pathologists. The location of these structures between parenchymal cells and blood vessels leads to the conviction that they have considerable physiologic significance, constituting a layer across which fluids, solutes, and macromolecules must pass. Understanding the nature and structure of basement membranes has been enhanced by the application of histochemical methods of study and electron microscopy. In an effort to evaluate further the structure of the basement membranes in the kidney, an investigation utilizing these methods has been carried out in rats of different ages. The primary object of the study has been to determine whether significant alterations occur in the structure and composition of the basement membranes of renal glomeruli, tubules and capillaries in the process of aging.

## Methods

Albino rats of the Sprague-Dawley strain were utilized. Three age groups were represented; 10 infant animals were 2 to 7 days old, weighing 15 to 25 gm.; 10 young rats were 2 to 6 months of age, weighing 75 to 150 gm.; and 10 old rats were 1½ to 2 years of age, weighing 300 to 450 gm. The rats were sacrificed by sudden cervical dislocation, and sections of kidney were obtained immediately. Thin slices were fixed in cold neutral buffered formalin for conventional histologic and histochemical investigation, and 1 cmm. blocks were fixed in a chrome-osmium solution [1] for electron microscopy. Paraffin sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), Alcian blue,[2] toluidine blue at pH 4, azure B [3] with and without ribonuclease, Mallory's trichrome, phosphotungstic acid-hematoxylin, and Verhoeff's elastic tissue stains. Sudan black B stain was also utilized on carbowax sections in some instances. The tissue blocks for electron microscopy were dehydrated in alcohol, embedded in methacrylate and sectioned on a Porter-Blum microtome with a diamond knife.[4] The sections were examined with an RCA EML microscope.

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## Observations

The glomerular capillary wall could be resolved into its various constituents by conventional microscopy, using histochemical methods. With azure B in ribonuclease-controlled sections, the general distribution in the cytoplasm of epithelial cells was ascertained. Stainable ribonucleoprotein did not form a very compact mass in the cytoplasm of individual cells, however, but appeared as a thin layer of granules on the outer surface of the capillary wall. Basement membranes and other cement substance were visualized best with the Alcian blue and PAS staining procedures, used singly or in combination. The combination of azure B with PAS provided an excellent means of visual distinction between cytoplasm and basement membrane. In the infant rat, there were observed a very faint PAS and Alcian blue-positive basement membrane, a proportionately greater amount of Alcian blue-stainable but PAS-negative mucopolysaccharide, and a relatively large amount of azurophilic intracyloplasmic substance (Figs. 1 and 2). In young rats the PAS-positive constituent of the basement membrane became more distinct and prominent, and the Alcian blue-staining cement and azurophilic cytoplasmic substances became less prominent (Fig. 3). In the old rats these changes were still more marked (Figs. 4 and 5), and the PAS-positive basement membrane seemed to be thicker and somewhat wrinkled. The azurophilic substance in the epithelium was less noticeable. The cytoplasm of the glomerular epithelium was found to contain PAS-positive material in the young and old rats, but this was much less prominent in the infant animals.

Electron microscopy demonstrated the fine structure of the capillary wall clearly. In infant rats epithelial and endothelial cells prominently occupied a major portion of the glomerulus. The cytoplasm of these cells was compact and contained many fine ribonucleoprotein granules. Basement membranes were clearly composed of 3 zones, inner and outer layers with little electron density, and a single central compact dense zone (Figs. 6 and 7). In young rats there was a slight thickening of the central layer, and moderate narrowing of the inner and outer zones (Fig. 8). In the old rats the central dense lamina composed the major portion of the basement membrane; inner and outer laminas were quite thin (Figs. 9 and 10). The relative thickness of the 3 laminas varied in different capillary loops but generally followed the pattern indicated. The epithelium in the young and old rats was attenuated and thin, but its trabeculas and pedicels were thicker, as compared with those of infant rats.

The parietal glomerular basement membrane was also increased in

electron density and thickness in the older rats, as compared with the infants (Figs. 6 and 11). There was a corresponding increase in the thickness and prominence of its PAS-positive component. At all ages, fibrillary structure was apparent in this region, while the dense portion of the visceral basement membrane was homogeneous and compact in all age groups.

Tubular basement membranes generally stained well with both PAS and Alcian blue. They were very thin, however, in the infant rat, and in this group the PAS stain was faint. The electron microscope similarly revealed a delicate and thin peritubular basement membrane (Fig. 12). Alcian blue-positive material in the region of the peritubular and interstitial tissue was quite abundant. With aging, the PAS-staining layer became thicker while the Alcian blue-stainable cement substance of the interstitial tissue was reduced. Comparably, by electron microscopy the tubular basement membrane was found to be thickened in the older animals. The intertubular connective tissue ground substance also contained a greater amount of PAS-positive substance in young and old rats as compared with newborn animals.

## Discussion

In all instances the basement membrane of the glomerular capillary loops was found to be of homogeneous density, with no evidence of a fine internal structure. On the other hand, the parietal basement membrane, in most instances, exhibited a fine fibrillary meshwork. Spiro[5] described an ordered structure of glomerular basement membrane with an array of filaments containing spaces somewhat less than 100 Å in breadth. This description apparently related to the capillary loop basement membrane; if so, we have not been able to confirm this observation. Pease,[6] in his classic description of the fine structure of the kidney, noted that the basement membrane had a homogeneous density, a feature confirmed by Bergstrand[7] and Yamada.[8] Pease[6] also described the dense central layer in the basement membrane and considered the inner and outer layers of less density to be cement substance. Our observations were in conformance with these and demonstrated, moreover, that with aging, the central lamina became thicker while the inner and outer less dense portions became attenuated. This process became apparent in the rat at about 3 to 4 months of age.

Differentiation of the various glomerular constituents (epithelium, basement membrane, and endothelium) is not easily made by light microscopy. It may, however, be accomplished through the use of histochemical methods that selectively demonstrate cytoplasmic ribonucleoprotein (azure B staining, controlled by ribonuclease digestion), and those that

specifically stain cement and basement membrane substance (PAS and Alcian blue). The combined use of Alcian blue and PAS reveals a separation of two extracytoplasmic components in the general region of the basement membrane. One of these is PAS and Alcian blue-positive, thought to be identical with the dense central lamina as seen in electron microscopy. Alcian blue-stained material which is PAS-negative apparently represents the inner and outer laminas with less electron density. However, the thinness of the inner layer adjacent to endothelium precludes the detection of Alcian blue-positive, PAS-negative substance in this region. There is, moreover, a poorly demarcated though nonetheless Alcian blue-positive, PAS-negative deposit on the outer side of the dense lamina. This would appear to represent a cement substance surrounding the foot processes and trabeculas of the epithelium. Because this substance has so little electron density, its outermost limit and distribution cannot be determined entirely by electron microscopy. It is probable that some of it extends between and may coat the epithelial pedicels and trabeculas.

There is no unanimity of opinion as to the derivation of the basement membrane and cement substance. These may represent products of fluids passing between endothelium and parenchymal cells. They may also be derived as secretory products of either epithelium or endothelium, or both. Since no connective tissue elements are demonstrable in the region of the glomerular capillary loops, these substances would not appear to be related to supporting or connective tissue. Since the portions of the basement membrane with less electron density lie as laminas adjacent to both the epithelium and the endothelium, it seems logical to consider them to be products of the contiguous cells. It is also compatible with its structural character to postulate that the dense central component of the membrane is a more compact and older form of the less dense cement substance. This reasoning would assume the origin of the basement membrane to be from both epithelium and endothelium, forming first as a less dense, PAS-negative and Alcian blue-stainable mucopolysaccharide. Later alterations, by compression and possibly through other physical or chemical modifications, lead to the development of the Alcian blue and PAS-positive central dense layer.

In this investigation, it has been possible to demonstrate a PAS-positive substance in the cytoplasm of glomerular epithelium. The azure B-PAS stains reveal an intermingling of azurophilic granules with more abundant PAS-positive material. This suggests that the PAS-positive component is intracytoplasmic and does not represent the carbohydrate encountered in the intertrabecular spaces of epithelial processes. The cytoplasmic carbohydrate may conceivably migrate from the epithelial

cell in the process of glomerular filtration. A counterpart to this phenomenon is thought to constitute the basis for the PAS-positive droplets discerned in renal tubular epithelium; these are believed to represent albumin (and presumably also carbohydrate) in the process of movement from the lumen to the peritubular capillaries.[9] An alternative possibility is that the PAS-positive glomerular epithelial substance may reflect the site of the production of basement membrane cement substance prior to its extrusion and deposition.

The significance of glomerular and tubular basement membrane thickening in the aging rat requires further evaluation. The part played by basement membranes in determining the rate and selectivity of filtration remains debatable. Hall[10] believed that the basement membrane was not a significant factor in determining relative glomerular impermeability to protein. He felt that the small size of the slit pores between epithelial pedicels constituted the factor which prevented the escape of protein. On the other hand, Pease[6] maintained that the slit pores were too large to have a restraining influence upon plasma albumin; he attributed the failure of albumin to escape into the glomerular filtrate to the relative impermeability of the membrane. Spiro[5] demonstrated the existence of focal defects in the glomerular basement membrane in a number of renal diseases associated with proteinuria. He attributed the increased permeability to protein to these defects, thus emphasizing the view that the relative permeability of glomeruli was dependent upon the structure and integrity of the basement membrane. In an investigation of the structure of capillaries, Bennett[11] considered that the density of the basement membrane favored the concept that capillary permeability was dependent upon the nature of the membrane. Greater knowledge of the molecular structure and physical characteristics of basement membranes is required to solve the problem.

The glomerular basement membrane has been shown to undergo thickening as hypertension develops in the rat subjected to choline deficiency at the time of weaning.[12] Whether this represents a cause or effect of the hypertension is debatable, however. Hypertension generally occurs in older individuals or may be related to some mechanism of glomerular injury causing thickening of the basement membrane. Moreover, the effect of glomerular basement membrane thickening as a cause of altered quantity and composition of glomerular filtrate needs further evaluation. There is an additional possibility that thickening of the membrane with increasing age may be related to an impairment of the free movement of macromolecules suspended in the body fluids. This could be a factor in the local deposition of large protein or lipid molecules in vessel walls as in atherosclerosis.

## Summary

An investigation of the basement membranes of the glomeruli, tubules and peritubular capillaries in the albino rat has been carried out. Comparative studies were made on animals shortly after birth, after weaning, and in late adult life, utilizing histochemical methods and electron microscopy. It has been possible to show that the PAS-staining component of the glomerular basement membranes increases in prominence with aging. This is accompanied by a reduction in Alcian blue-positive, PAS-negative substance in and around the basement membrane area. The epithelial cytoplasmic component of the glomerular capillary wall is prominent immediately after birth, becoming attenuated and thin as age advances. These alterations are paralleled by changes demonstrable with electron microscopy. At all ages the basement membrane is characterized by a central dense zone, the lamina densa, and zones of lesser density adjacent to both epithelial and endothelial elements. The dense zone is PAS-positive, and the areas of lesser density PAS-negative but Alcian blue-positive. There is an increase in the thickness of the dense central lamina and thinning of the less dense inner and outer zones of cement substance as the animal ages. A PAS-positive substance was demonstrated in the cytoplasm of the glomerular epithelial cells.

It is suggested that the Alcian blue-positive, PAS-negative, and electron light zones of the basement membrane may represent the earliest manifestation of a cement substance, derived from the epithelium and endothelium, and that this becomes altered physically or chemically to form the dense central lamina which is PAS and Alcian blue-positive.

It was pointed out that thickening of the basement membrane in the glomeruli and tubules with increasing age could be related to the development of hypertension, to the alteration in quantity or quality of glomerular filtrate, or to the abnormal deposition of macromolecular particles in the walls of blood vessels.


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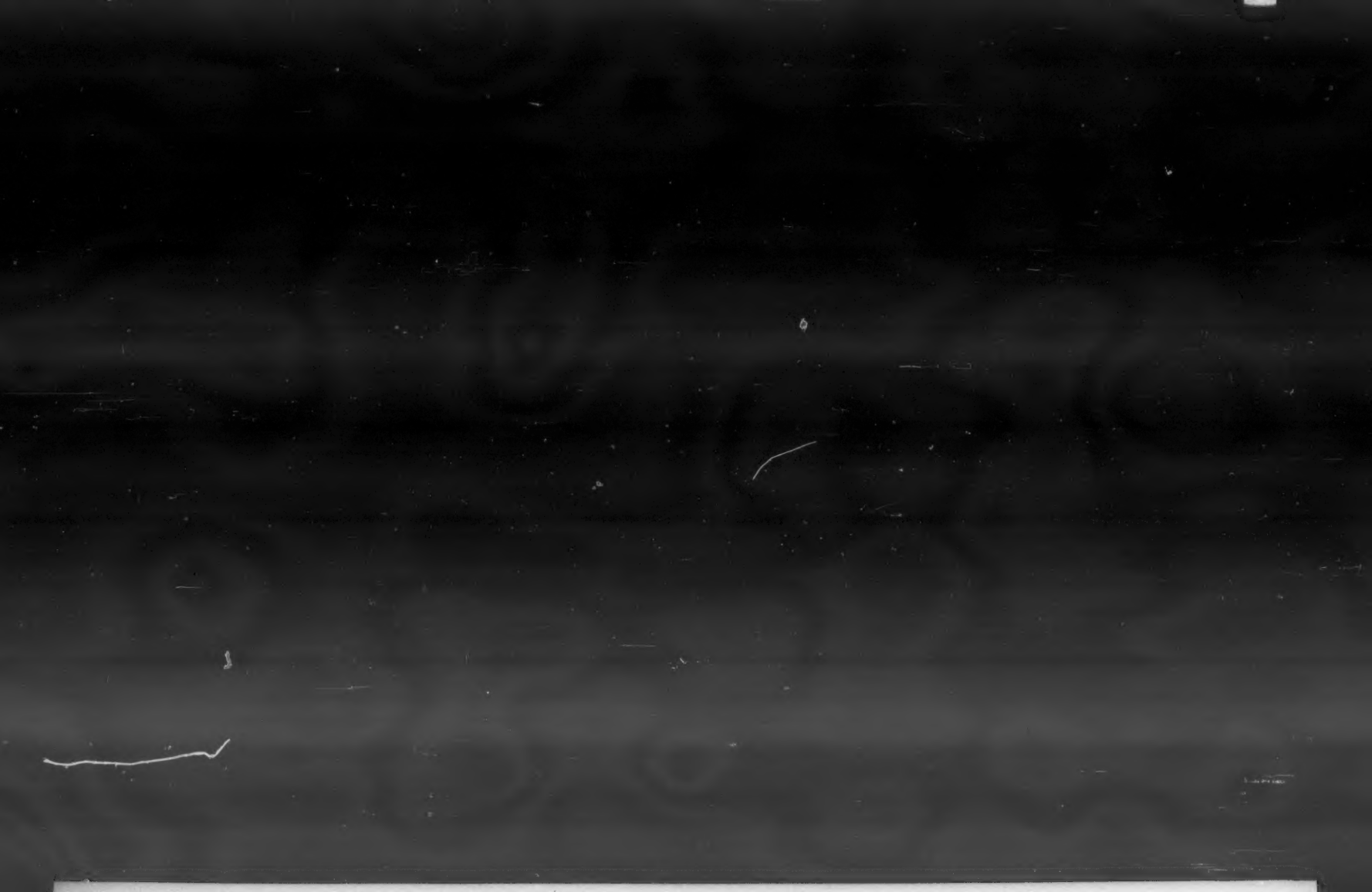
*[Illustrations follow]*

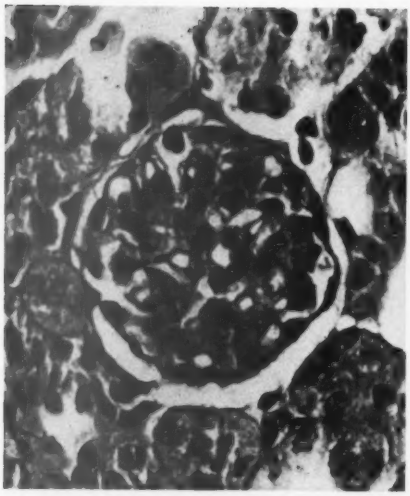
## Legends for Figures

Fig. 1. Periodic acid-Schiff (PAS) and Alcian blue-hematoxylin stain of the kidney of an infant rat. The basement membrane (arrow) of the capillary loops has a dark PAS and Alcian blue-positive, fine linear component (black in photograph). Surrounding this dark portion is a faint Alcian blue-positive and PAS-negative layer which is not sharply delineated (gray zone around basement membrane line in photograph). × 750.

Fig. 2. PAS-azure B stain of the kidney of an infant rat. The fine PAS-positive basement membrane is visible (black in photograph). The azure B-staining cytoplasm of the epithelial cells is visible as poorly demarcated, gray, finely granular material around the epithelial cell nuclei (arrow). Azure B-staining ribonucleoprotein granules are also noted in the tubular epithelial cells as fine gray granules in the photograph; PAS-positive droplets in the epithelial cell cytoplasm appear as small black particles. × 750.

Fig. 3. PAS-azure B stain of the kidney of a young rat. The PAS-positive basement membrane is more prominent than in the kidney of baby rats. Azure B-stainable cytoplasm of the epithelial cells of the glomerulus is less abundant. × 750.





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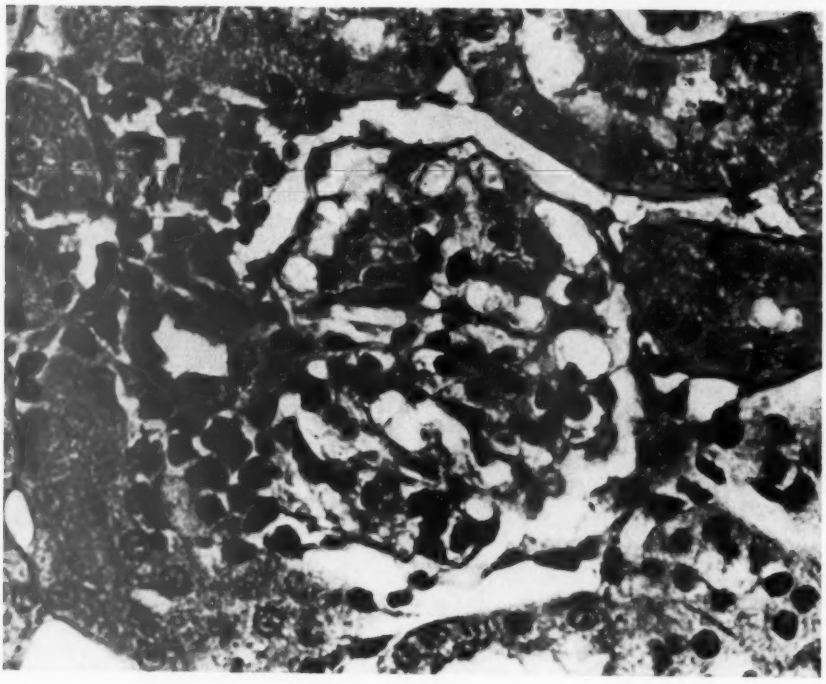

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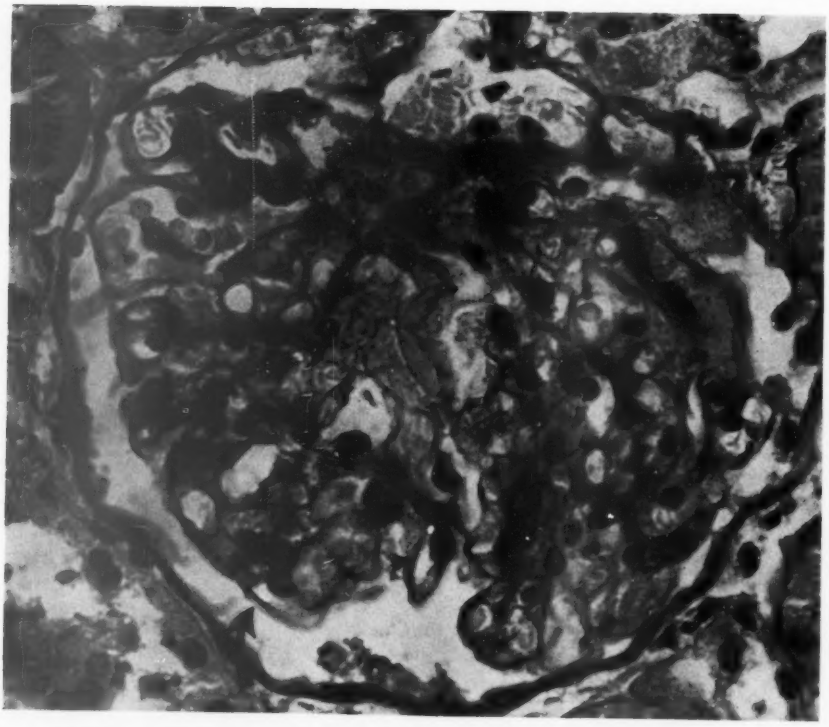
FIG. 4. PAS and Alcian blue-hematoxylin stain of the kidney of an old rat. The thicker basement membrane is visible as a black line (PAS and Alcian blue-positive). A small amount of Alcian blue-positive and PAS-negative cement substance can be seen in the photograph as gray material (arrow) located around the denser linear portion. Darker gray to almost black condensations around the nuclei of the epithelial cells were strongly PAS-positive and Alcian blue-negative. These are interpreted as being intracytoplasmic in the epithelial cells. × 750.

FIG. 5. PAS-azure B stain of the kidney of an old rat. There is a decreased amount of azure-staining epithelial cytoplasmic granules visible in the photograph as gray, slightly granular material around the black (PAS-positive) basement membrane (arrow). The darker gray, smudgy areas around the nuclei of the epithelial cells are PAS-positive material in the epithelial cytoplasm (arrow). × 750.



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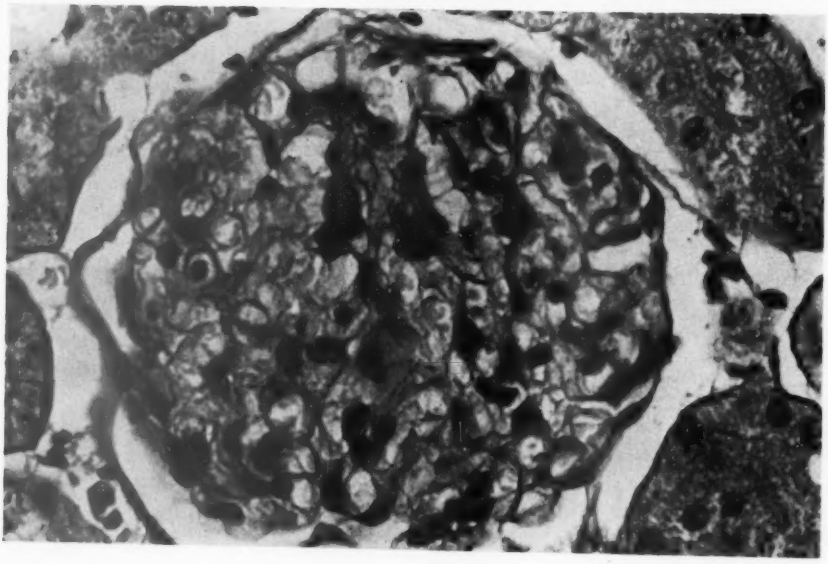

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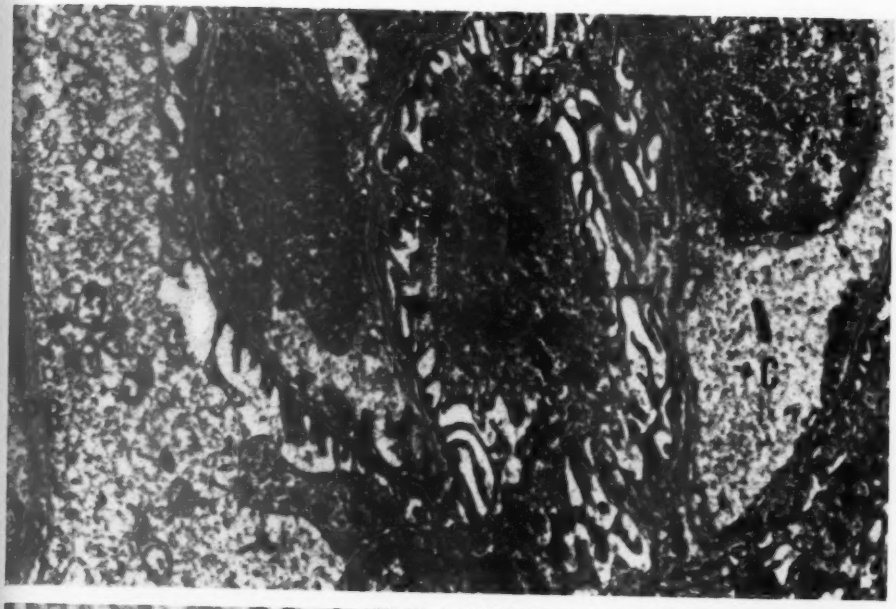
Figures 6 to 12 are electron micrographs.

FIG. 6. Glomerulus of an infant rat. The capillary loops (C) have narrow lumens, and the lining endothelial cells (E) fill a considerable portion of the capillaries. The epithelial cells (EP) have abundant, electron-dense cytoplasm, and the trabeculas and pedicels (arrows) are delicate. The basement membrane (B) is clearly divisible into a thin, delicate, central dense portion, and rather wide, clear zones located on either side of the denser lamina. The parietal basement membrane (PB) is noted to be much thicker than that around the glomerular capillaries and has a loose-textured fibrillary composition. × 8000.

FIG. 7. Kidney of an infant rat, showing the thin dense central zone and the lighter zones (arrows) of the basement membrane (B) of a glomerular capillary loop. × 18,000.

FIG. 8. Capillary loop of a glomerulus of the kidney of a young rat. The central dense portion of the basement membrane (B) is somewhat thickened as compared with that in the infant rat. Epithelial cell trabeculas and pedicels (arrows) are thicker. × 18,000.

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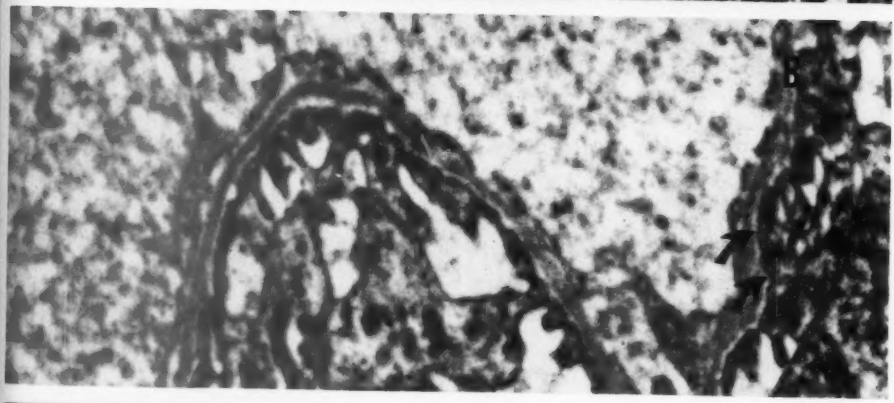


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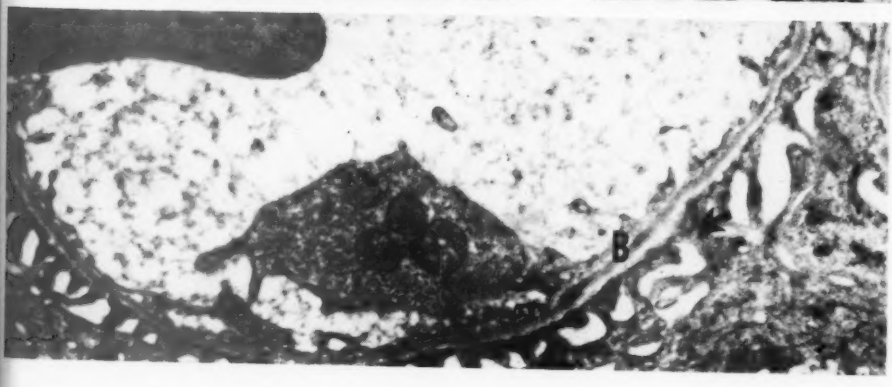


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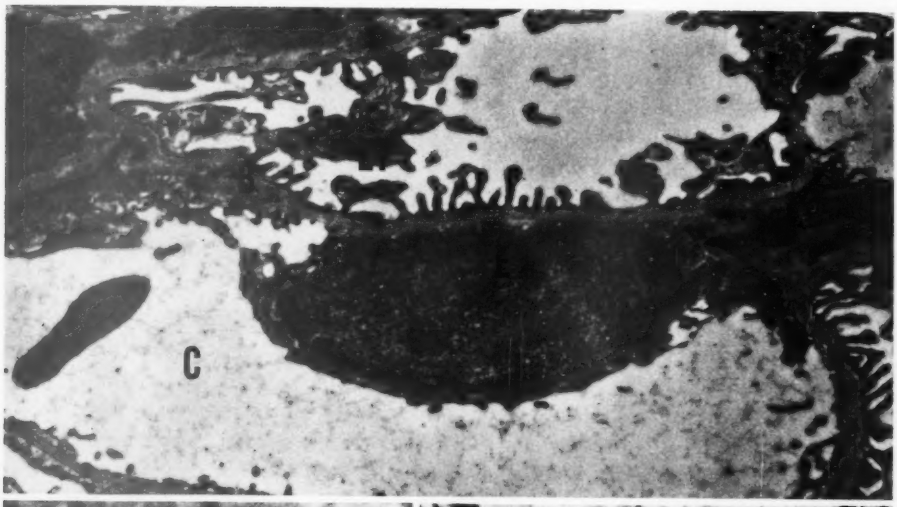
FIG. 9. Glomerulus from an old rat. The wider capillary lumens (C) and proportionately less cytoplasm of epithelial (EP) and endothelial (E) cells are apparent. The basement membrane (B) is more dense and homogeneous. The central denser portion is wider than in infant and young rats, and the lucid zones on either side of the dense lamina are considerably reduced. × 8000.

FIG. 10. Glomerulus of an old rat. The thick, dense, central portion of the basement membrane (B), and the narrow lucid zones (arrows) of cement substance can be seen. Epithelial (EP) and endothelial (E) cell cytoplasm can be seen on either side of the basement membrane. The trabeculas (T) and pedicels of the epithelial cells are thicker than in infant and young rats. × 18,000.

FIG. 11. Kidney of an old rat. A very fine basement membrane is visible around an adjacent intertubular capillary (arrow) and is distinct from the epithelial basement membrane. A portion of the thick parietal basement membrane (PB) of an adjacent glomerulus is seen, revealing a loose, fibrillary structure. × 8000.

FIG. 12. Portions of the proximal convoluted tubules (TU) of an infant rat. A thin, dense lamina (B) is present around each tubule. No definite lucid zones are apparent around this tubular basement membrane. × 8000.

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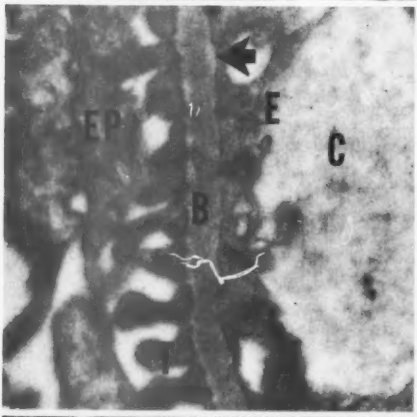


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# STUDIES IN EXPERIMENTAL ENDOCARDITIS

## I. Production of Valvular Lesions by Mechanisms Not Involving Infection or Sensitivity Factors

Alfred A. Angrist, M.D.; Masamichi Oka, M.D.; Komei Nakao, M.D., and Jeanne Marquiss, M.D.

*From the Department of Pathology, Albert Einstein College of Medicine of Yeshiva University, New York, N.Y.*

Many experimental studies have implicated allergic sensitization or local infectious mechanisms in the formation of valvular vegetations. Though a number of organisms have been used, staphylococci and streptococci have been employed most often. Jones and Carter [1] have gathered the references demonstrating the role of allergy, streptococci or both; their review also noted that valvular lesions followed anaphylactic shock and injections of whole or fractionated horse, bovine, duck or pig serums and streptococcus toxin. Thomas, Brunson and Smith [2] and Stetson [3] used meningococcus toxin to induce a local Shwartzman phenomenon in the valve. Strehler [4] utilized anti-aorta serum.

It is difficult to invoke the phenomenon of sensitivity to explain valvular lesions that follow a single injection of an antigen, particularly when they occur rapidly and before allergic sensitivity or antibody can be demonstrated.[2,3] Jones and Carter [1] noted this fact and also produced lesions with tested nonallergenic material, such as neutral polysaccharide, vitreous humor and gastric mucin. Rinehart and Mettier [5] observed that scurvy played a role in the production of valvular lesions. There was no evidence that sensitivity was instrumental in the appearance of valvular vegetations following such nonspecific forms of stress as high altitude,[6] arteriovenous shunts,[7,8] pitressin [9] or papain and amphetamine sulfate.[10]

The high incidence of nonbacterial vegetations with many sensitizing substances and nonantigenic forms of stress would seem to exclude local infection as the common denominator in the induction of their initial valvular lesions. These considerations, in conjunction with the frequent occurrence of vegetations of this nature on human valves [11,12] without evidence of infection or antigen-antibody mechanisms, as in "terminal endocarditis" in patients with uremia or cachexia, and the frequent

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transitions encountered in all forms of endocarditis,[13] favor the hypothesis that local sensitivity or infection is not generally a factor in the evolution of the early phases of valvular vegetations.[14]

The present series of experiments was undertaken to develop methods of producing vegetations and investigating their early stages. Rats were subjected to various types of nonspecific stress for these purposes.

## Material and Methods

Adult male and female rats of the Sprague-Dawley strain, weighing approximately 200 gm., were used. These were maintained on Purina Rat Chow and water, unless otherwise stated.

The physiologic stresses cited below were purposefully selected to avoid the factors of infection, sensitization and immunization. In each experimental group there were usually 5 control animals kept under identical conditions without the addition of the particular stress. In addition, there was a special control group of 56 rats maintained with all forms of stress reduced to a minimum.

### *Normal Rats*

The occurrence of some alterations in occasional parallel control rats prompted a more detailed investigation of a larger series of normal rats kept under conditions of minimum stress. These animals were isolated from 1 to 12 weeks in individual cages and kept in an air-conditioned room at 72° F. and 50 per cent humidity. The 56 rats handled in this fashion were of different ages and were grouped by weight as follows: males: 5 less than 150 gm.; 16, 151 to 300 gm.; 11, more than 300 gm.; females: 6 less than 150 gm.; 18 between 151 and 500 gm. These served to establish the range of spontaneous lesions in the valves of this strain of rats at different ages, sizes and sexes.

### *Nonspecific Forms of Stress*

1. Tumbling. Sixteen rats were placed in a tumbling machine 3 times a week. Two hundred tumblings were administered initially; there were increments of 100 until 1,000 tumblings per session. The rats were sacrificed after 4 to 35 such treatments.

2. Exposure to cold. Sixteen rats were placed in a cold room at 4° C., 4 hours a day, 4 days a week. The animals were sacrificed after 36 to 131 exposures.

3. Combined stress of high altitude and cold. Sixteen rats were placed in a cold chamber (4° C.) at a simulated altitude of 25,000 feet for 4 hours a day, 4 times a week. The rats were sacrificed after 36 to 131 exposures.

4. Combined stress of parabiosis and castration. Fourteen pairs of females were joined by the technique of Brunster and Meyer.[15] Simultaneously a bilateral ovariectomy was performed on one member of the pair. Animals were maintained in this state for 8 to 21 days. Estrogen in large doses constitutes a form of stress which results in hypertrophy of the adrenals.[16] It was for this reason that one of the parabiotic partners was castrated at the outset of parabiosis. The procedure resulted in a marked stimulation of the follicle-stimulating hormone (FSH) in the noncastrated partner, with hypertrophy of the ovaries and the occurrence of a high estrogen level.[17]

All rats were sacrificed by concussion. The hearts were removed promptly, surveyed rapidly under the binocular microscope and fixed in cold formol-calcium. After fixation, the valves were re-examined with the binocular microscope. Gross alterations or vegetations of the valves or chordae tendineae were recorded. Selected areas were taken for histologic study. Lesions were occasionally noted in the fixed valve which were not seen as readily in the fresh tissue. Paraffin sections were stained with hematoxylin and eosin, phosphotungstic acid-hematoxylin (PTAH), the periodic acid-Schiff (PAS) procedure, dilute eosin and methylene blue at various levels of pH, toluidine blue, Alcian blue, and an Alcian blue-PAS-van Gieson combination.

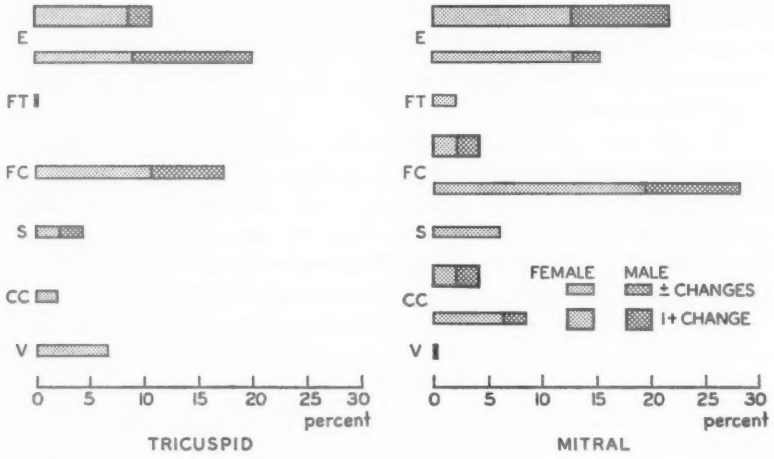
## Results

The combined gross and microscopic observations are presented in the form of graphs. The charted alterations represent minimal rather than true values. Serial sections of entire valves were not attempted for practical reasons. Slides prepared from different blocks in the same valve or from contiguous areas deeper in the same block showed considerable variation. It is certain that some lesions which could not be detected in the gross were not sectioned and, therefore, were not included in the final tabulations.

In the valves of control rats kept under the usual conditions of normal care, the changes encountered were focal edema and thickening. No vegetations were encountered. The lesions observed in the special control group kept under ideal conditions are detailed in Text-figure 1. Those showing valvular alterations also exhibited some other abnormality at necropsy (i.e., myocarditis, pneumonia, or nephrosis). In none were these lesions more than moderate (1+); the majority were minimal (±). Vegetations of the fresh platelet variety were found only on the tricuspid valve in two animals, one with nephrosis. Focal edema was the most frequent and most marked change encountered. Occasional valves exhibited focal surface cellularity characterized by hypertrophic and

swollen fibroblasts. Changes in the staining quality of collagen were minimal. Figures 1 to 8 demonstrate the range of alterations encountered in this special control group.

Experimental stresses induced a variety of lesions in and on the valves and chordae (Text-figs. 2 to 5). In order to avoid undue complexity,




TEXT-FIGURE 1. Control group.

All bar graphs are constructed so that the total length of each column represents the incidence percentage of a given valvular lesion in a particular group. The width of each column in turn indicates the degree of severity of the alterations. Minimal lesions (±) are charted only for a special group of normal controls maintained under conditions of minimal stress in order to give the complete range of reaction in these valves. This is also the only chart in which the sexes are indicated separately. There were no variations in the lesions related to the sex of the animals.

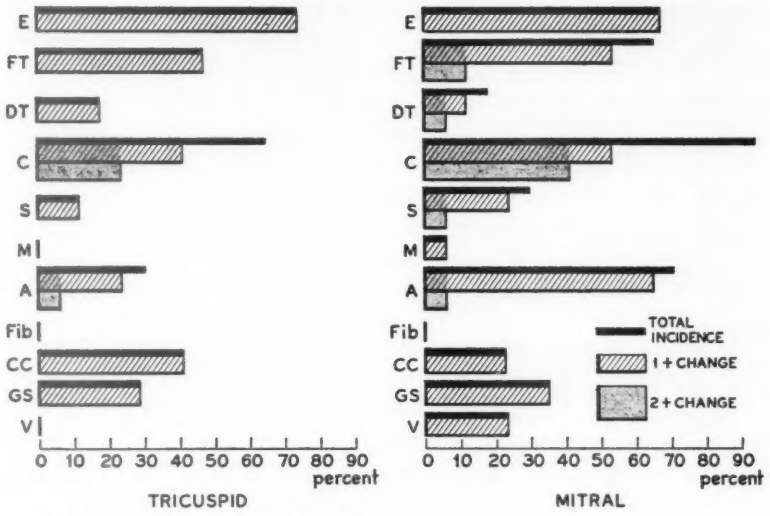
Lesions of moderate and marked severity are designated 1+ and 2+, respectively. The dense black line in the charts pertaining to the experimental groups indicates the overall total incidence of moderate and marked alterations. By overlapping the 2+ and 1+ columns, a graphic representation of the total valve changes is provided.

Key for all graphs:

FE = focal edema
E = diffuse edema
T = thickening
FT = focal thickening
DT = diffuse thickening
C = increased number of cells throughout the valve
S = surface localization
FC = focal clusters of cells
M = monocytic infiltration
A = increased number of Anitschkow cells
Fib = fibroblastic proliferation
CC = collagen changes, including eosinophilia, separation and vacuolation of collagen fibers
GS = swelling of the ground substance
Fd = fibrinoid
V = vegetations

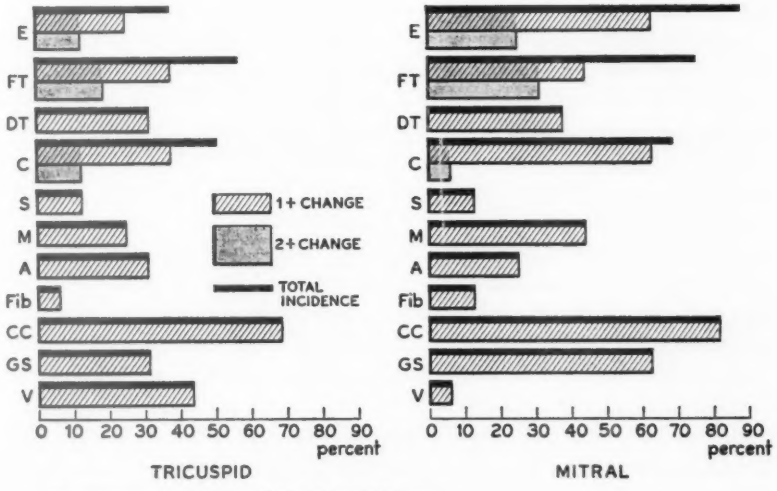
minimal (±) alterations are not indicated. The lesions varied even under seemingly similar conditions.

The lesions in rats subjected to tumbling are given in Text-figure 2. It will be noted that the valves showed edema, focal or linear fibrous

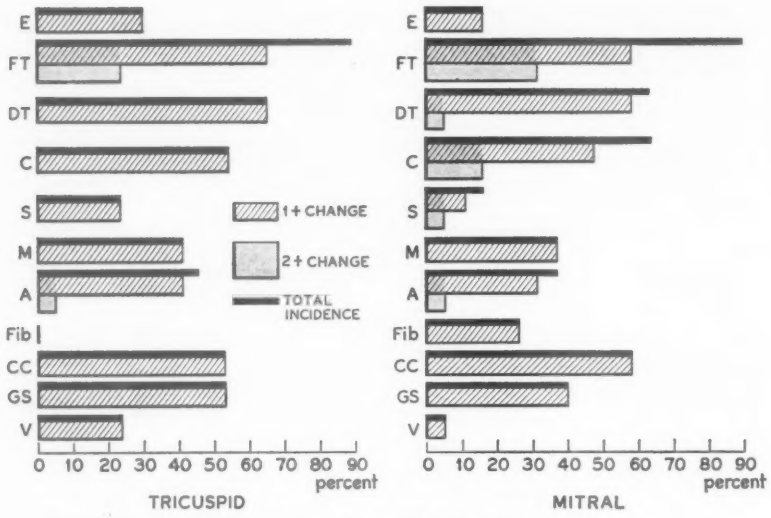



TEXT-FIGURE 2. Group subjected to tumbling.

thickening, increase in cellular content, mainly Anitschkow cells, a few monocytes and mast cells, swelling of the ground substance and increased eosinophilia and spreading of collagen fibers. In general, except for the edema, the alterations were more marked for the mitral valve. Platelet vegetations were limited to this valve (Figs. 9 and 10).

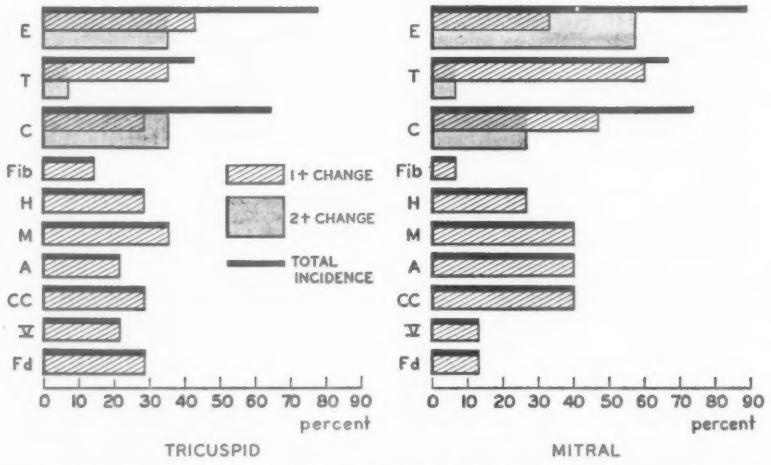



TEXT-FIGURE 3. Group subjected to cold room.




TEXT-FIGURE 4. Group subjected to high altitude in cold room.

In animals exposed to intermittent cold, the edema, fibrous thickening, increased number of monocytes, Anitschkow cells and fibroblasts, changes in the collagen and the ground substance were more marked (Text-fig. 3 and Figs. 11 and 12). In this instance also the lesions were more severe in the mitral valve. In one case, spontaneous bacterial endocarditis affected both the mitral and tricuspid valves. All other




TEXT-FIGURE 5. Group subjected to parabiosis with castration of one parabiont.

fresh vegetations were composed, in the main, of platelets and were limited to the tricuspid valve.

The rats exposed to both high altitude and cold exhibited focal and diffuse fibrous valvular thickening (Fig. 14), surface infiltration by monocytes, Anitschkow cells and fibroblasts, and alteration of the connective tissue. The lesions appeared equally in both valves, mitral and tricuspid (Text-fig. 4). Vegetations were also present on both valves in a relatively small percentage of the animals and were of the fresh platelet variety.

The valves in rats subjected to parabiosis and castration of one parabiont showed the most advanced lesions (Text-fig. 5 and Figs. 13, 15 and 16). The mitral valve was more often the seat of marked distortion. In addition to edema, thickening and fibroblastic proliferation, an increase of cellular content, particularly monocytes, and evidence of mitotic activity were very prominent. Changes in the collagen and ground substance were conspicuous, and fibrinoid deposit was observed in both the valve substance and the vegetations.

Special stains for connective tissue were not revealing; the alterations seemed essentially to represent edema and distortion of collagen. The PTAH stain demonstrated remarkably little fibrin in the fibrinoid lesions or vegetations. Alcian blue-staining stroma was increased in staining intensity in most of the valves. In those in which edema was a prominent feature, the intensity was diminished. Extracellular PAS staining was limited to fibers, apparently of collagenous nature. The cytoplasm of some of the larger mononuclear cells, particularly among the castrated parabionts was also PAS-positive. No explanation can be offered for this, although some of the cells probably represented altered mast cells.

The multiple experimental conditions and the small series of rats used in any one group require explanation. Obviously, no statistical evaluation is being attempted. The primary objective was to achieve a technical procedure that would induce early valvular lesions with a degree of consistent uniformity of timing in order to permit their morphologic investigation.

The adrenals in the rats subjected to chronic stress were hypertrophied, and there was a general correlation between the degree of hypertrophy and the severity of the valve changes. An index of hypertrophy was derived by taking the ratio of adrenal weight to body weight of each animal as the numerator, with the ratio derived in a similar fashion for the same size and sex as indicated in the tables of Gray and Addis[18] for normal rats, as the denominator. Hypertrophy was most marked in young rats exposed to intermittent cold and in rats subjected to parabiosis and estrogen stimulation. In the latter group the average of

the indexes (ratio of adrenal weight to body weight) was 1.2659 as compared to 1.0442 in the special control group. In the rats subjected to parabiosis only, the average index of hypertrophy was 1.0592. In those exposed to continuous cold, the average index was 1.1852. For young rats (average weight, 125 to 200 gm.) exposed to intermittent cold, the average index was 1.5182. In older rats (400 to 500 gm.) this index could not be derived because the standard tables of Gray and Addis gave no data for rats weighing more than 419 gm.

## Discussion

These studies confirm the observations of others that valvular lesions, including vegetations, can follow different forms of stress in the absence of infection, sensitization or an immunization factor.[1,5-10] The lesions induced suggest an alteration of the valve connective tissue, particularly of its ground substance.

The occurrence of lesions in the valves of normal control animals is significant, and the alterations in the experimental rats must be evaluated with these spontaneous lesions as base lines. The implication is that the valve tissue is quite labile and susceptible to the minor degrees of stress occurring in normal animals. The ready production of valvular lesions by many forms of stress lends further support to such a hypothesis.

The frequent occurrence of fresh vegetations, particularly of the platelet variety, and the low incidence of organized stages of these lesions requires explanation. Apparently the platelet vegetations can undergo rapid dissolution and absorption and do not often show progressive organization. They resemble the platelet vegetations described by Grant, Wood and Jones[19] in the pockets and on roughened surfaces of human valve leaflets. These lesions were thought to favor localization of organisms on the valve, constituting a nidus for bacterial endocarditis. The valvular thickening found in the experimental animals was not ascribable to organization of the platelet thrombi; the thickening appeared to follow the interstitial alterations.

The most common early vegetations were platelet thrombi, which were preceded by changes within the valve proper. The most common alteration in the latter region was characterized by a focal or diffuse edema and an increase in fixed tissue elements. Increased eosinophilia of collagen and intensification of Alcian blue staining were not directly related to the overlying vegetations. Fibrinoid deposit and reactive cellularity were found consistently in relation to the vegetations in the parabiotic group, as were alterations in the staining of the collagen. In only one instance did fibrinoid in the vegetation appear to stem from the under-

lying collagen as suggested by Allen.[20,21] It would appear that vegetations may have more than one mechanism of origin, i.e., adhesion of platelets, fibrin precipitation, or extrusion of altered valve substance.

Some of the variations in the valvular reactions are undoubtedly attributable to differences in the ages of the animals. Although the experimental groups consisted of rats weighing approximately 200 gm. at the outset, the experiments were of variable duration, and the animals were thus of different ages at the time of necropsy. The age factor did appear to have some bearing on the alterations of collagen and thickening of the valves. In the tumbling series the age factor was specifically investigated by dividing the animals into a younger group weighing 200 gm. or less and a more mature group weighing approximately 350 gm. In the control series, the younger rats showed fewer spontaneous alterations, but in the young experimental group there was a heightened susceptibility to the production of lesions.

The varied character and intensity of the lesions in rats subjected to stress under identical conditions cannot be explained. An inherent ability to withstand stress may constitute an individual quality, determined by genetic and endocrine factors or past exposure to stress. The possibility of an endocrine factor is suggested by the hypertrophy of the adrenals encountered in the experimental animals. The well known effect of steroid and pituitary hormones on normal and abnormal connective tissue also favors this possibility.[22,23] It is conceivable that a sensitivity state and systemic infection may act locally on the valvular stroma, at least initially, through the medium of an induced endocrine aberration. A unified concept that the initial alteration in the valve constitutes a common denominator in all vegetations and their transitions as encountered in human disorders of this nature, has been proposed.[11,14,15]

## Summary

Heart valve lesions may appear following different forms of stress. The alterations are varied in nature, intensity and frequency. They are characterized by alteration of the valvular ground substance, abnormalities of collagen, the deposition of fibrinoid, fibrous thickening, and an increase in fixed tissue cell content; the latter vary and include Anitschkow cells, fibroblasts, monocytes and mast cells. Orientation in palisade-like manner to the surface is a noticeable feature. Additional features are swelling of the endothelium, and the appearance of platelet or thrombotic vegetations. Only occasionally is there spontaneous bacterial contamination. Similar lesions with considerably less severity appear in normal and pretreated controls.

The significance of these alterations as representing fundamental

preparatory changes to the evolution of nonbacterial and bacterial vegetative valvular disorder as encountered in human disease requires further investigation and thought.

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We wish to thank Dr. Helen W. Deane for making some of the special preparations.

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*[Illustrations follow]*

## Legends for Figures

Except where indicated, photographs are from sections stained with hematoxylin and eosin.

Fig. 1. Five-week-old female control rat (84 gm.). Animal kept under optimal conditions for one week with minimal stress. Mitral valve is thin and transparent. The spherical shadow at its base represents an air bubble trapped beneath the cusp.

Fig. 2. Mitral valve of a 2-month-old female rat (182 gm.) kept under optimal conditions for one month. The fibrosa and spongiosa are normal. × 140.

Fig. 3. Eight-week-old male rat (191 gm.) kept under optimal conditions for 2 weeks. The tricuspid valve is normal throughout except for minimal edema in the chordae tendineae on the left, extending to the papillary muscle.

Fig. 4. A 12-week-old female rat (206 gm.) kept under optimal conditions for 8 weeks. Tricuspid valve has normal structure. × 110.

1

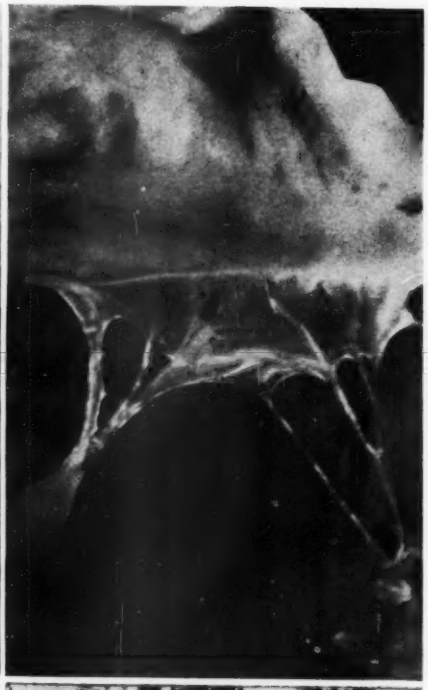

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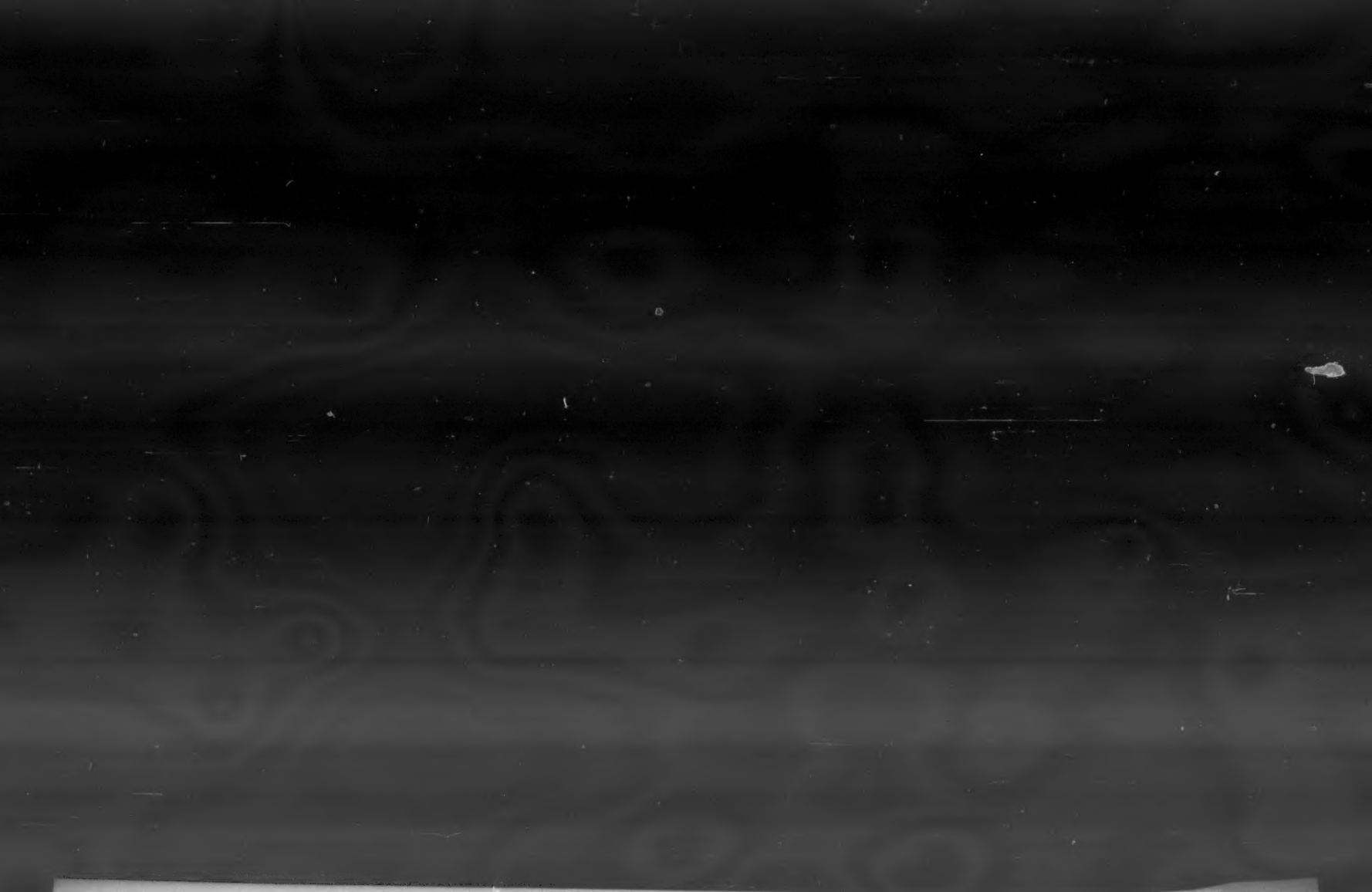
FIG. 5. Section of tricuspid valve in rat whose mitral valve is seen in Figure 1. There is minimal (±) edema in the lower half of the valve. Myocarditis characterized by a diffuse mononuclear infiltration is present in the regional muscle. × 180.

FIG. 6A. Heart valve in a 13-week-old male rat (401 gm.) kept under optimal conditions for 9 weeks. There is edema of the tricuspid valve and its chordae tendineae. A confluent linear platelet and fibrin vegetation is seen on the atrial surface of the valve (lower right arrow), and another large vegetation extends to the left onto some chordae (upper, larger arrow). The kidneys of this rat were large and pale, and the seat of nephrosis with marked dilatation of collecting and convoluted tubules.

FIG. 6B. Valve shown in Figure 6A. A portion of the platelet vegetation at the tip of the valve appears in the lower right. × 90.

FIG. 7. Aortic valve in a 16-week-old male rat (447 gm.) kept under optimal conditions for 12 weeks. Diffuse edema (1+) and increased cellularity are prominent at the tip (upper left). The cellular content consists of fibroblasts and occasional monocytes and mast cells. The myocardium also exhibits a cellular infiltration. × 140.

FIG. 8. Valve illustrated in Figures 6A and 6B, showing moderate (1+) edema with an attached platelet and fibrin vegetation. × 180.



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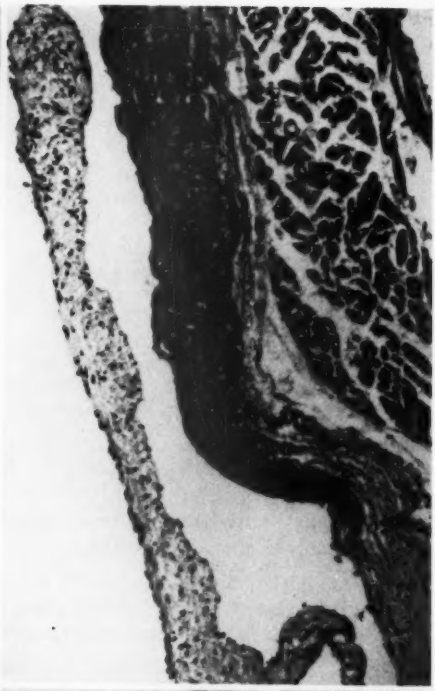

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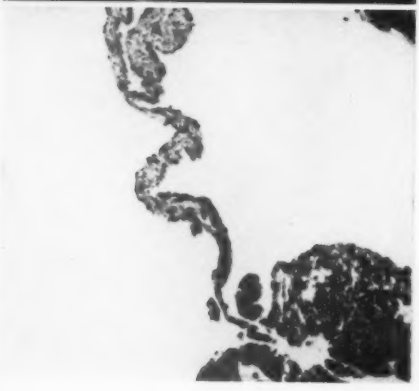

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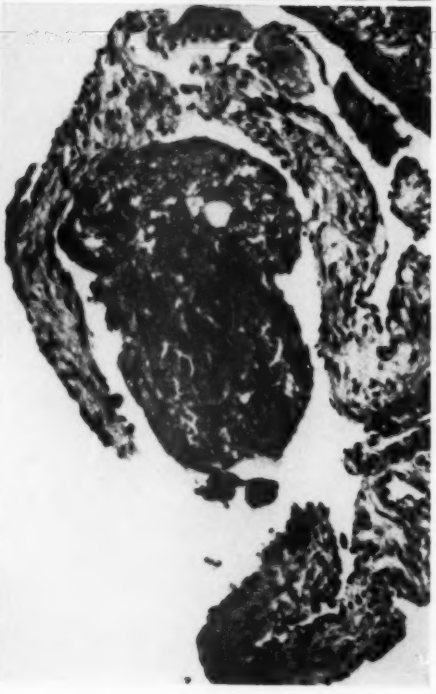
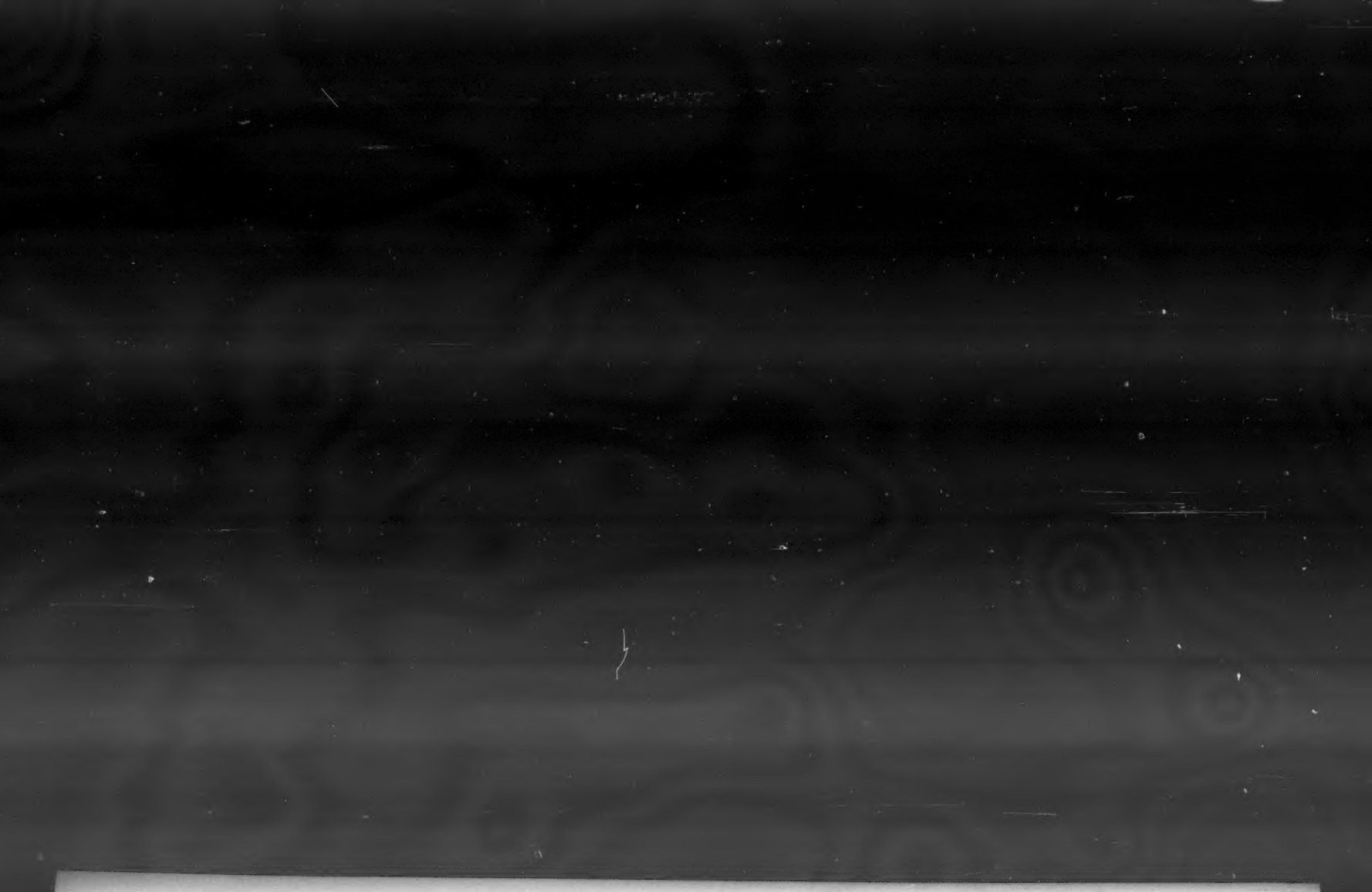


FIG. 9. Rat subjected to 35 tumbling periods. There is diffuse thickening of the mitral valve (at tip of dissecting needle). A small, protuberant vegetation (arrow) is seen on the short cusp and extends a short distance along the chordae.

FIG. 10. Rat subjected to 35 tumbling periods. Aortic valve showing considerable edema (2+), focal eosinophilia and distortion of collagen. Cellularity is composed chiefly of Anitschkow cells, fibroblasts, rare lymphocytes and monocytes. There is surface localization at the tip, focal grouping and a tendency to perpendicular orientation. × 130.

FIG. 11. Rat exposed to cold (4° C.) 82 times. Mitral valve shows adherent blood protein or protruding valve substance (arrow), edema and a few cellular elements. Phosphotungstic acid-hematoxylin stain. ×180.

FIG. 12. A spontaneous bacterial vegetation on the mitral valve. Rat exposed to cold 82 times. The vegetation (black arrow, lower right) contains altered platelets, fibrin and bacteria. At its base at the level of the valve surface, there is an inflammatory exudate, with neutrophils predominating. On the ventricular surface of the valve opposite the bacterial vegetation are 2 small vegetations (hollow arrow) without exudation. These suggest nonbacterial vegetations. PTAH stain. × 150.



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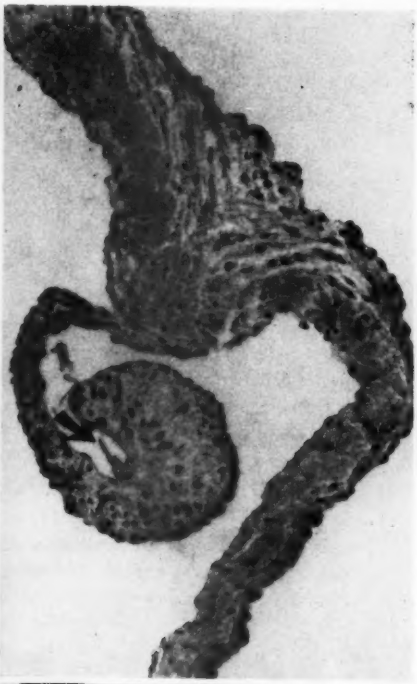

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FIG. 13. Parabiotic rat 14 days after operation. Tricuspid valve shows diffuse and marked thickening, with a "billowing sail" appearance. Microscopically this valve revealed marked cellularity and some adherent fibrinoid. See Figure 16.

FIG. 14. Rat after 45 exposures to reduced atmospheric pressure (equivalent to 25,000 feet) and cold (4° C.). Mitral valve is thickened and shows edema, increased cellularity with palisading of fibroblasts separated by edematous ground substance. Occasional monocytes and alteration of collagen are apparent. No vegetation is manifest. × 130.

FIG. 15. Partner of castrate rat after 10 days of parabiotic union. Tricuspid valve shows edema and fibrous thickening. Nonbacterial vegetations appear on the valve (arrow) and chordae tendineae above the papillary muscle. Fibrinoid was found within and upon the valve.

FIG. 16. Parabiotic rat 8 days after operation. Tricuspid valve shows marked thickening, cellularity and adherent fibrinoid (arrow). The cellular infiltrate in the valve consists of compactly placed monocytes, hypertrophied fibroblasts, mast cells and Anitschkow myocytes. Only a few neutrophils and lymphocytes were present. Mitotic figures are frequent in the cellular infiltrate. × 90.

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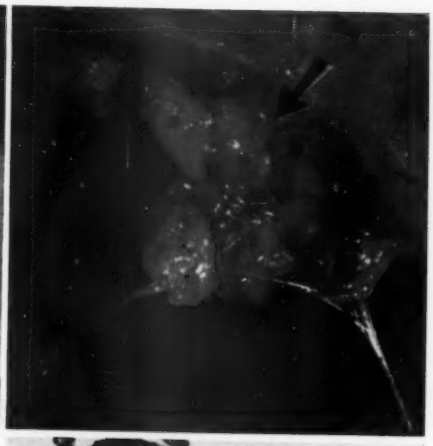

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# COLONIC HISTIOCYTOSIS IN CHILDREN

## Report of a Form Resembling That Seen in Whipple's Disease

David T. Rowlands, Jr., M.D., and Benjamin H. Landing, M.D.

*From the Departments of Pathology and Pediatrics of the Children's Hospital, Children's Hospital Research Foundation, Cincinnati General Hospital, and the College of Medicine, University of Cincinnati, Cincinnati, Ohio.*

The purpose of this paper is to describe the occurrence, in the colonic mucosa of two children, of histiocytes with histochemical properties similar to those observed in Whipple's disease. A histologic and histochemical comparison is made between the lesions in these two patients and those in a previously reported adult with Whipple's disease.[1] The implications of the findings are discussed in relation to possible genetic and pathogenetic mechanisms.

In 1907, Whipple[2] reported striking pathologic alterations in the small intestine and mesenteric lymph nodes of a 36-year-old male. Mononuclear and polynuclear giant cells were noted in the mucosal stroma and submucosa. The lacteals of the intestine contained masses of neutral fat and fatty acids, and the mesenteric nodes contained the same fat deposits, but in greater amounts. Whipple named this condition "intestinal lipodystrophy." Since his report, at least 77 cases have been described,[3] and the disorder has come to be considered a well defined entity.

In 1949, Black-Schaffer[4] demonstrated, in 4 patients with Whipple's disease, that the substance in the large histiocytes of the intestine stained strongly with the periodic acid-Schiff (PAS) stain. Identical deposits were found in most of the giant cells in the mesenteric lymph nodes. The substance did not take the Best's carmine stain, but stained faintly with mucicarmine. These features, and evidence of water-insolubility, led him to the conclusion that the intracellular deposit was a glycoprotein. The same 4 cases were again reported by Hendrix, Black-Schaffer, Withers and Handler,[5] who reviewed other recorded cases. They concluded that the clinical course was fairly uniform, but not pathognomonic. Two features characterized the pathologic lesion, lipogranulomatosis of the mesenteric lymph nodes, and the presence of macrophages (histiocytosis) in the lamina propria of the small intestine. In one instance the proximal portion of the colon was also affected. In addition, it was felt that the absence of anatomic evidence of lymphatic obstruction was a significant negative feature.

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In 1950, Plummer, Russi, Harris and Caravati [6] collected 34 cases of Whipple's disease from the literature. The patients were predominantly males (81.6 per cent) and were, for the most part, 30 to 60 years of age. Recent reports of the occurrence of Whipple's "intestinal lipodystrophy" in siblings [3,7] have suggested the possibility that this disorder, the basic nature of which remains unknown, is genetically determined. That all reported cases were in whites and the great majority were males, Puite and Tesluk [7] considered evidence of a genetic disorder. Moreover, in view of the fact that most patients were over the age of 25 years, these authors suspected "a clinically 'silent' intestinal defect existing frequently for years before it becomes symptomatic."

To date, there are apparently no recorded examples of asymptomatic Whipple's disease, namely, of characteristic lesions discovered as incidental features at operation or necropsy. Recently, the authors have observed histiocytes in the lamina propria of the colon of two children with no clinical evidence of Whipple's disease. The histochemical and morphologic features appeared to be similar to those considered pathognomonic of Whipple's disease. Search of sections from 96 successive necropsies of infants and children has failed to reveal similar cells. In addition, an investigation of tissues from 28 necropsy and surgical cases representing a variety of metabolic and infectious diseases which might cause colonic histiocytosis yielded negative results.

## Case Reports

*Case 1 (Children's Hospital, C-58-124)*

A 1½-year-old adopted white male was in good health until 4 weeks prior to admission, when he developed diarrhea which lasted approximately 10 days. Another child in the family at that time had infectious hepatitis, and the patient was given a prophylactic injection of gamma globulin. Twelve days before admission he suddenly developed high fever (105° to 106°F.), which lasted 2 days, and was associated with generalized lymph node enlargement. He improved after treatment with chloromycetin. On the day of admission he awoke suddenly from his afternoon nap, apparently with agonizing abdominal pain and with almost continuous vomiting.

On admission, the temperature was 97.4° F., the respiratory rate was 24 per minute, and the blood pressure was 120/70. The child was well developed and nourished. Abdominal skin had decreased turgor; no masses were felt.

The morning after admission the abdomen was distended and tympanitic. Volvulus of the small intestine with gangrene from the duodenum to the mid-transverse colon was found at operation. The volvulus was reduced, but the patient expired shortly after operation.

At necropsy, begun 1½ hours after death, the body was well developed and nourished. The abdominal cavity contained a small amount of bloody fluid. The intestine was unrotated, with the colon extending vertically up the midline; the mesenteric root was short and transverse, and there was infarction of the intestine from the duodenum to the mid-colon. The distal portion of the colon appeared normal. The heart was enlarged (88 gm.). Both lungs were increased in weight (right, 149 gm.;

left, 125 gm.), with large zones of atelectasis and edema. The spleen exhibited prominent white pulp, and the kidneys were congested. Mesenteric lymph nodes were edematous and hemorrhagic. Examination of the cranial contents was not permitted.

Microscopic examination confirmed the gross findings in the heart, lungs, and spleen. Malpighian follicles also showed evidence of toxic degeneration. The pylorus and duodenum were the seat of nonspecific chronic enteritis and early mucosal necrosis. The remaining portions of the small intestine and the proximal colon displayed diffuse hemorrhagic necrosis. Sections of the distal colon revealed many macrophages in the lamina propria. In hematoxylin and eosin (H and E) stained sections, these were filled with opaque, amphophilic cytoplasmic granules (Figs. 1 to 3). The outer layer showed only acute serositis. The pancreatic islets were increased in size and in number. The adrenals displayed a decrease in lipid, with cystic areas in the zona glomerulosa. The mesentery was edematous and contained recent hemorrhage. The blood vessels and lymphatic vessels were distended, and the nodes were hemorrhagic and necrotic.

*Case 2 (Children's Hospital, C-58-85)*

An 11-month-old white male infant was the product of a normal pregnancy (birth weight, 8 pounds, 8 ounces); both parents were apparently healthy. An older sibling died at 20 months of age of febrile convulsions and "encephalitis."

One day before admission the patient's eyes began to water. On the morning of admission he developed fever, and purulent exudate was noted in the eyes. Right-sided "twitching" was observed shortly before admission. On admission the temperature was higher than 108° F. (rectal), the pulse was 140 per minute, the respirations were 48 per minute, and the blood pressure was 80/60. The child was well developed and nourished; there was marked cyanosis. Generalized twitching was now observed. His abdomen was soft, and no organs were palpable.

Treatment resulted in a reduction of temperature to 101° within 4 hours. Penicillin and chloromycetin were given. However, shock developed, and the patient, despite supportive measures, expired on the day following admission.

Necropsy examination was begun 10 hours after death. The child was well developed, and approximately normal in size for his age. The jejunum and ileum were collapsed, and Peyer's patches were prominent. The appendix was normal. The colonic wall was of the usual thickness and gross appearance, and the lumen contained soft fecal material. The spleen was markedly enlarged (44 gm.), with scattered dark red hemorrhages.

The liver was moderately enlarged (365 gm.) and had a dark red-brown surface with patchy pale areas. Cervical, mediastinal, para-aortic and mesenteric lymph nodes were of normal size. The brain was significantly enlarged (1,210 gm.) and bulged when the dura was opened. There was no evidence of hemorrhage, and no pressure cone was found. On section, the white matter in all areas of the cerebrum was thin.

Microscopically, there was a scanty lymphocytic infiltration at the gastroesophageal junction, as well as toxic alteration of lymphoid nodules in the gastrointestinal tract. The colon contained large PAS-positive histiocytes in the lamina propria (Figs. 4 and 5). The pancreatic islets were slightly increased in number and size. The zona glomerulosa of the adrenals had a high lipid content while that of the zona fasciculata was reduced. The brain was the seat of focal neuronal alteration and gliosis. Although the lesion was considered to represent acute edema and neuronal necrosis superimposed on old nerve cell loss and failure of myelination, the possibility of a familial "abiotrophy" was not eliminated. It was not possible to obtain necropsy material from the sibling.

*Case 3*

The patient whose tissues were used for histochemical comparison was previously reported by Sailer and McGann.[1] He was a 45-year-old white male who had been ill for 5 years prior to death. At necropsy, the abdomen was distended, and approximately 4,000 cc. of cloudy yellow fluid was found in the abdomen. The mesenteric nodes were enlarged, firm and elastic. The serosa of the gastroenteric tract was dull and lusterless. The mucosa was not noteworthy.

Microscopically, villi of the small intestine revealed marked widening and blunting by a granulomatous process extending into the submucosa. The outstanding cytologic feature was the presence of closely packed large histiocytes containing fine to coarsely granular, pale-staining droplets (Fig. 6); between these cells were large clear spaces appearing to be dilated lymphatics. Mesenteric lymph nodes exhibited a sponge-like pattern and contained multinucleated giant cells, and foamy histiocytes of the type found in the small intestine.

## Histochemical Studies

Histochemical analyses of colonic sections from the 3 cases are shown in Table I; PAS, pectinase-PAS, toluidine blue, pectinase-toluidine blue, glucuronidase-toluidine blue, hyaluronidase-toluidine blue, Sudan IV on Zenker-fixed paraffin sections, and direct aldehyde-fuchsin stains were performed. Additional stains were carried out as indicated in the table.

Tissues fixed in both Zenker acetic acid and in 10 per cent formalin were available. It was noted that Zenker-fixed tissue provided much stronger staining reactions, so that most of the investigations were made with tissue fixed in this solution. For case 3 it was necessary to use paraffin blocks prepared at the time of necropsy, since no wet tissue remained; the record did not state the fixative employed.

Incubation of paraffin sections of formalin-fixed tissue in 2.5 per cent potassium dichromate solution with 5 per cent acetic acid for 24 hours at 37° C. provided stronger reactions with both PAS and hematoxylin and eosin (H and E) stains than did formalin fixation alone. However, a

more pronounced increase in staining power was obtained by placing formalin-fixed tissue in either a saturated solution of mercuric chloride or in a solution of 2.5 per cent potassium dichromate and 5 per cent

TABLE I

HISTOCHEMICAL ANALYSES OF COLONIC SECTIONS

| Stain | Case 1 C-58-124 | Case 2 C-58-85 | Case 3 Sailer and McGann [1] |
|---|---|---|---|
| Periodic acid-Schiff (PAS) | Purple-red | Purple-red | Purple |
| PAS-glucuronidase | Purple-red | | |
| PAS-hyaluronidase | Purple-red | | |
| Digested PAS (saliva) | Purple-red | | |
| Pectinase PAS | Purple-red | Purple-red | Purple-red |
| Toluidine blue | Metachromatic | Metachromatic | Weakly metachromatic |
| Pectinase-toluidine blue | Metachromatic | Metachromatic | Negative |
| Glucuronidase-toluidine blue | Metachromatic | Metachromatic | |
| Hyaluronidase-toluidine blue | Metachromatic | Metachromatic | |
| Crystal violet * | No metachromasia | | |
| Sudan IV, frozen section * | Negative | | |
| Sudan IV, paraffin section, Zenker fixative | Negative | Negative | Negative |
| Sudan black B * | Negative | | |
| Direct aldehyde-fuchsin | Purple | Purple | Weak |
| Acid permanganate aldehyde-fuchsin | Purple | | Weak |
| Alcian blue | Blue | | Blue |
| Luxol blue | Negative | | |
| Acid fast | Red | | Negative |
| Coupled tetrazonium | Negative | | |
| Direct dihydroxy-dinaphthyl-disulfide | Negative | | |
| Thioglycolate-dihydroxy-dinaphthyl-disulfide | Negative | | |
| Gram stain | Purple | Purple | |
| Gomori methenamine silver | Positive | Positive | |
| Neuraminic acid stain (Diezel) | Probably negative | Probably negative | |

* Formalin fixed tissue.

acetic acid for 24 hours, and embedding it in paraffin after washing in water for an additional 24 hours.

In the two children, H and E stained sections of the colon revealed large numbers of histiocytes confined to the lamina propria. The nuclei were large and vesicular. The cell border was sharply demarcated, and the cytoplasm contained an abundance of granular amphophilic substance (Fig. 2). With the PAS stain this appeared as particles of irregular size and shape, entirely filling the cytoplasm (Fig. 5). None was

found free in the stroma of the lamina propria. In the adult (case 3) the intracellular substance had an appearance similar to that seen in the children. An identical PAS-positive substance lay free within the extracellular stroma (Fig. 6). Similar cells were also found in the mesenteric lymph nodes, and occasionally in the spleen, but this was the extent of systemic involvement.

The PAS-positive material in case 1 was not reduced on incubation with saliva for 0.5 hours, nor by incubation with glucuronidase or hyaluronidase for one hour. The glucuronidase test was performed using Ketodase (beta-glucuronidase, Warner-Chilcott Laboratories, Inc.) in a concentration of 5,000 units per cc. with the pH adjusted to 5.0 and the temperature at 37° C. The hyaluronidase test used Wydase (hyaluronidase, Wyeth Laboratories, Inc.) in a concentration of 150 turbidity reducing units per cc. at a temperature of 37° C. and at a pH of 5.7.

Metachromasia was observed with toluidine blue in all 3 cases. A toluidine blue extinction study was performed on the tissue from patient 1; the end point of the toluidine blue staining was observed to be between pH 2 and 3. No metachromasia was observed with crystal violet.

## Discussion

In the recent past, histochemical analysis has been applied to the histiocytes of Whipple's disease by a number of workers. Christie and Galton[8] reported positive PAS and Hale stains, and faintly positive Meyer's mucicarmine stain. Best's stain for glycogen was negative, and they were unable to demonstrate metachromasia with toluidine blue. Upton[9] reported a necropsy of a 54-year-old male who, in addition to the usual lesions in the small intestine and in lymph nodes, showed alterations in the colon adjacent to the ileocecal valve. Upton reported positive results with mucihematin, mucicarmine, PAS (also after diastase and hyaluronidase digestion), and Hale stains, and found that the substance was metachromatic with toluidine blue. The metachromasia was not affected by hyaluronidase digestion, but it was lost after acetylation, as was the PAS-positivity. Other reactions, including methyl violet for metachromasia and a variety of fat stains, were negative. Jones, Benson and Roque[10] investigated a 49-year-old female with Whipple's disease. They demonstrated that the material in the cells was water-insoluble, basophilic, metachromatic, PAS-positive, and not digested by saliva or hyaluronidase. Aniline blue stained the intracellular substance and the adjacent connective tissue to the same degree. Fat stains of the cells were negative, except for a few fine droplets. Fisher and Whitman[11] examined a 27-year-old female, and performed a series of histochemical studies on the histiocytes in mesenteric lymph nodes. They described PAS-positive

cells, with no change in staining after diastase or hyaluronidase. Fat stains on frozen sections revealed a few lipid-containing histiocytes. Thionine (pH 4) gave no metachromasia, and negative results were obtained with a variety of other stains. Casselman, Macrae and Simmons,[12] in studying two cases of Whipple's disease, found that basophilia could not be demonstrated by the methylene blue extinction test at any pH between 1.5 and 9.5. In addition, the metachromatic staining by toluidine blue was extremely labile in ethanol. The granules resisted hydrolysis by hyaluronidase, amylase, and pectinase and were positive with Hale's stain.

All studies appear to agree that the substance within the histiocytes in Whipple's disease is PAS-positive and resists digestion by saliva and hyaluronidase. Similar staining properties were observed in our 3 patients. Metachromasia with toluidine blue was very definitely present in both children, and it was present weakly in the adult control case. The variability of this property noted in the literature has led to considerable misunderstanding. It may well be that more than one mucopolysaccharide is present in the cells, and that certain fixatives, including formalin, or certain embedding techniques, remove one or more so as to eliminate metachromasia. Further weight is added to the concept of multiple mucopolysaccharides by the acid-fastness (basophilia) of the substance in the two children, and lack of this property in the adult when the tissues were originally reported and at the time of their re-examination. The intensification of the reactions by the use of Zenker's acetic acid solution on slides from formalin-fixed tissue supplies additional emphasis. In all cases the intracellular substance was negative to fat stains.

Whipple's disease, at least in the symptomatic form, is commonly considered to be a disease of middle age. Zampi and Graev[13] have described a 3-month-old infant male as having this condition. However, the failure to demonstrate PAS-positive material in the cells infiltrating the wall of the intestine, and the presence of an eczematoid rash and severe pulmonary disorder suggest that the patient actually had Letterer-Siwe disease. Fernandez Luna and Latienda,[14] in a 17-month-old child, demonstrated large amounts of fatty material within cells, but apparently did not perform a PAS stain. Peterson and Kampmeier[15] in 1951 reported two cases (their cases 2 and 3) in patients age 13 and 12 respectively. In the first of these, according to the authors, ". . . fat-filled phagocytes extended through the muscularis," and the histiocytes in their other patient were also described as fatty. In view of the now generally accepted concept that histiocytes free of lipid but containing mucopolysaccharides are pathognomonic of Whipple's disease, it is felt that none of these can be accepted as instances of Whipple's disease in children.

The fact that the disease apparently does not become manifest before adult life, and frequently occurs late in life, does not indicate that it is not of genetic origin. Puite and Tesluk[7] in 1955 reported 4 cases of Whipple's disease among which were 2 siblings. They were of the opinion that the disease is hereditary in nature, and that the primary defect is a metabolic one localized to the intestinal mucosa. They indicated that if a genetic factor is involved, it must be "dominant with great variability in expressivity and penetrance." The occurrence of well documented cases in women probably eliminates the possibility of sex-linked recessive inheritance.

Gross and co-workers in 1959 presented a second instance of the occurrence of Whipple's disease in siblings. A review of the family history of these patients revealed that both mental illness and diabetes mellitus had occurred in some of the relatives. Two sisters had diabetes. In addition, another sister developed schizophrenia, and a brother committed suicide at the age of 36 years. The authors suggested that, despite the relatively small total experience with Whipple's disease, and the generally poor family histories available, the fact that at least 6 reported patients had relatives with diabetes mellitus might be more than chance occurrence.

The significance of the cells observed in the children we are reporting, with the same morphologic and tinctorial properties as those seen in Whipple's disease, cannot be stated with certainty. The cells may represent an early stage of the disease, or the genetic carrier state; they may represent a similar but not identical metabolic disorder; or they may represent an entirely different and merely superficially similar process. The observations reported suggest that careful scrutiny of relatives, especially children, of affected patients may lead to more complete understanding of the disease. Also, since these cells were found in the colons of the two children, it may be that colonic biopsy in suspected cases, and of asymptomatic relatives of affected patients, would be rewarding. In addition, careful search for characteristic cells in the lymph nodes, liver, spleen, and blood smears of patients with sprue-like syndromes might serve to identify obscure forms of this condition.

As concerns the mechanism of accumulation of the polysaccharide substance in the children examined, several possibilities can be suggested. The material may be absorbed from the lumen of the intestine as the result of a defect in the mucosal epithelium, or it may be produced by alteration in the ground substance of the lamina propria, with subsequent phagocytosis. Absorption from the intestine is suggested by the histochemical similarity to intestinal mucin. Derivation from some dietary substance cannot be excluded, but the apparent absence of any

historically identifiable common dietary content in reported cases is against the concept. The finding of the interstitial substance may, on the other hand, lend support to the suggestion of Peterson and Kampmeier [15] that the basic feature of Whipple's disease is an alteration of connective tissue components.

Additional possibilities are that the histiocytes in the lamina propria synthesize the accumulated substance themselves, or that the mucus-secreting epithelium develops reversal of functional polarity and secretes mucus into the lamina propria. The usual restriction of the abnormal histiocytes to the intestine and draining lymph nodes appears to deny the possibility of a basic metabolic defect of the reticuloendothelial system generally.

## Summary

Infiltration of the colonic lamina propria in two children by histiocytes with histochemical features observed in Whipple's disease is described. This finding lends support to the concept that Whipple's disease is genetically determined but is clinically silent until adult life. The histochemical investigations described confirm previous reports that the histiocytes in Whipple's disease contain polysaccharide, but not lipid, and suggest that at least two polysaccharides are present, one metachromatic and relatively soluble, and one not metachromatic and relatively insoluble. The superiority of Zenker acetic acid to formalin fixation for investigation of these cells is stressed, and the possible value of biopsy of colonic mucosa for diagnosis of the disease is emphasized. Such biopsy of relatives and children of affected persons may well aid in clarifying the mechanism of the disease. The studies described do not permit identification of the source of the polysaccharide material.

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## LEGENDS FOR FIGURES

FIG. 1. Colon of case 1. Faintly granular histiocytic cells are stained by hematoxylin and eosin (H and E). × 160.

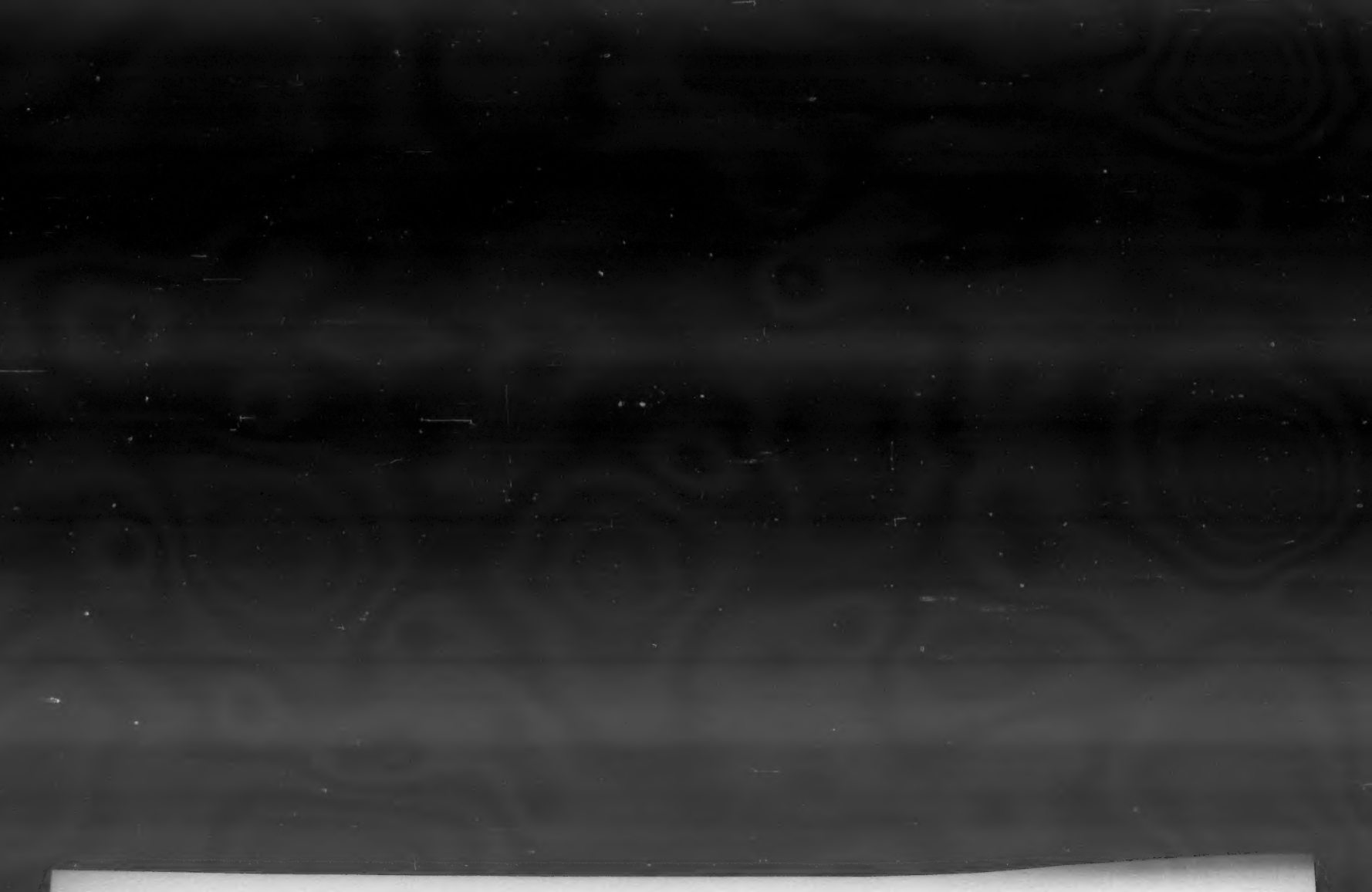
FIG. 2. Colon of case 1. The granular nature of the intracytoplasmic substance is evident with H and E staining. × 650.

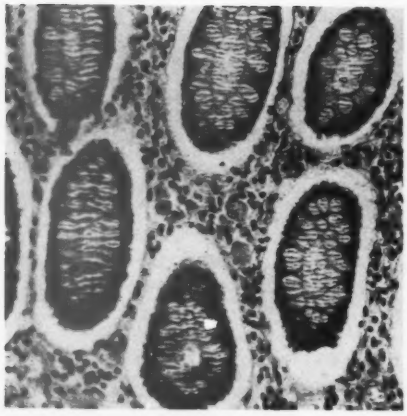
FIG. 3. Colon of case 1. Periodic acid-Schiff (PAS)-positive cells appear in abundance. × 160.

FIG. 4. Colon from case 2. PAS-positive cells are scattered diffusely through the lamina propria. × 160.

FIG. 5. Colon from case 2. The PAS-positive cells lie within the lamina propria. The particulate nature of the substance is clearly manifest. × 650.

FIG. 6. Small intestine from adult with Whipple's disease. The intensely staining PAS-positive substance is largely intracellular, but appears in extracellular location as well. × 650.





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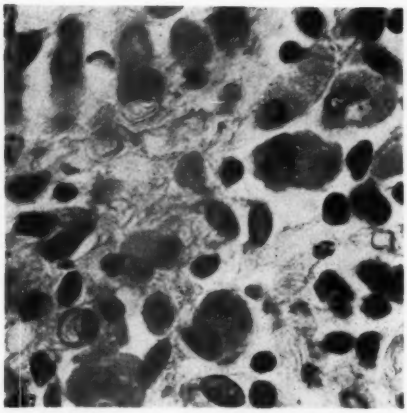

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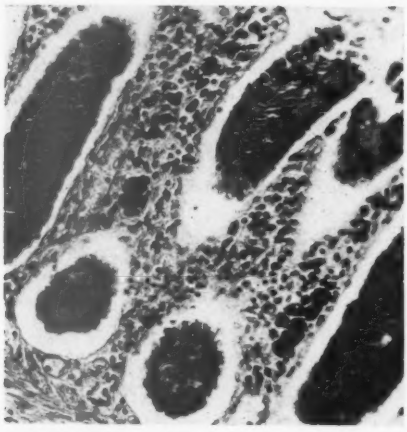

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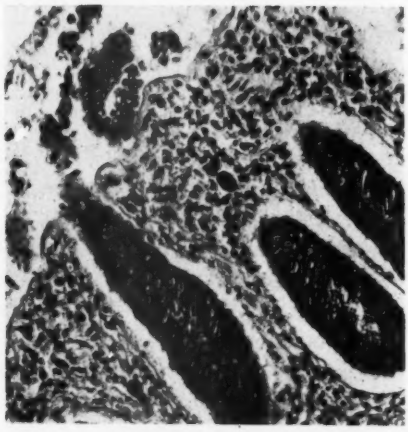

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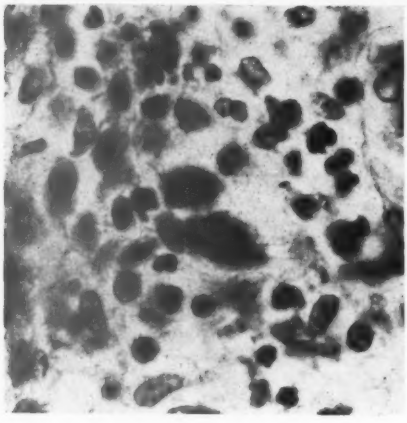

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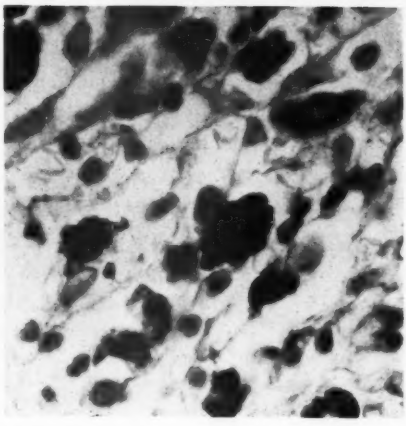

6

# EXPERIMENTAL THYROIDITIS IN RABBITS, GUINEA PIGS AND DOGS, FOLLOWING IMMUNIZATION WITH THYROID EXTRACTS OF THEIR OWN AND OF HETEROLOGOUS SPECIES

KORNEL L. TERPLAN, M.D.; ERNEST WITEBSKY, M.D.; NOEL R. ROSE, PH.D.; JOHN R. PAINE, M.D., PH.D., AND RICHARD W. EGAN, M.D.

*From the Departments of Pathology, Bacteriology and Immunology, and Surgery, of the University of Buffalo School of Medicine, and the Buffalo General Hospital, Buffalo, N.Y.*

For the past 6 years, the effects of active immunization of experimental animals with thyroid extract have been investigated in our laboratories. Extracts have been procured from homologous and heterologous species. A method for preparing comparatively undenatured and purified tissue extracts was developed.[1] Various immunologic procedures were tested in order to demonstrate thyroid-specific antibodies in the blood of immunized animals.[2,3] In the course of these studies the antigenic nature of crude saline extract of rabbit thyroid was clearly recognized in rabbits. Rabbit thyroid antibodies were demonstrated in the circulating blood following intradermal injection of minute amounts (0.05 ml.) of thyroid extract combined with the complete Freund adjuvants. These antibodies were organ-specific and reacted with rabbit thyroid extract. It was found that thyroidectomized rabbits, injected with autologous thyroid extract, produced antibodies which reacted with the autologous extract as well as with thyroid extracts obtained from other rabbits. The antigen content in the thyroid of immunized rabbits was considerably reduced in comparison to that procurable from the glands of nonimmunized animals. This reduction seemed to correlate with the titer of circulating antibody in the immunized animal.[4] In the thyroids of the immunized rabbits, histologic alterations were observed concerning which a brief preliminary description was given.[5] The resemblance of the histologic pattern in experimental thyroiditis to that of chronic thyroiditis in man has been discussed recently.[6,7]

It is the purpose of this paper to provide a detailed and inclusive analysis of the combined data obtained from investigations in the rabbit, dog, and guinea pig, carried out during the past 6 years. The relationship between auto-antibody titer and alterations in the thyroid gland suggested a direct interdependence. It is the present purpose to determine

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whether or not a correlation exists among the following factors: number of injections, intervals and duration, titer of the circulating antibody, and histologic alteration in the thyroid.

## Methods

The methods have been described in detail in earlier publications [1-8] and will only be summarized here.

The thyroid glands were excised, quickly frozen in dry ice and stored in this state. In some experiments, hemithyroidectomy was performed in order to investigate the effect of autologous inoculation upon the remaining gland tissue. In other instances, glands from many animals, sacrificed by exsanguination, were pooled. Before use, gland substance was thawed, trimmed of extraneous tissues, minced, and homogenized with an equal amount (w/v) of ice cold saline (buffered with phosphate salts to pH 7.2), using a Teflon homogenizer of the Potter type. After brief centrifugation, the crude extract was combined with an equal volume of Freund adjuvant and introduced intradermally into the footpads of the animals. Each inoculum contained 0.05 ml. of the emulsion.

As noted below, many different immunization schedules were used. On occasion, a purified thyroglobulin, prepared by Dr. S. Shulman, was injected instead of the crude extract.

The circulating thyroid antibodies in the serum of experimental animals were measured by means of precipitation, complement fixation and tanned cell hemagglutination tests. The latter procedure is a modification of the test originally devised by Boyden.[8] It consisted of treating human erythrocytes with highly diluted tannic acid and coating the tanned cells with thyroid extract. The coated tanned red blood cells were added to serial dilutions of the antiserum. The titer of the antiserum was measured as the greatest serum dilution showing definite agglutination.

Samples of all or part of the thyroid gland and of control tissue from various organs were immediately fixed in buffered formaldehyde, embedded in paraffin and stained with hematoxylin and eosin. Other staining methods used on occasion were Masson's trichrome, periodic acid-Schiff, Perls's Prussian blue and the Ciaccio stain [9] for lipids. All sections were examined without knowledge of immunologic data. The thyroid tissue available for histologic study was, in general, adequate for numerous sections. In a few instances, following hemithyroidectomy, part of the remaining lobe was also used for determining its antigen content, leaving less material for histologic study. In all cases, however, cross sections in the vertical or transverse axis of the thyroid were available.

The thyroid alterations were graded in accordance with the extent

of the interstitial inflammatory reaction and the reduction in size and colloid content of the follicles. The lesion designated as one plus (+) consisted of a few small interstitial infiltrates of lymphoid cells, and, occasionally, of a few fibroblasts with slight fibrosis unrelated to the lymphocytic infiltrates; these were usually seen about small thyroid follicles. The bulk of the thyroid tissue was normal, and there was no conspicuous desquamation of cells into the lumens of the follicles. Lesions designated as two plus (++) were moderate to severe in intensity and were characterized by prominent inflammatory infiltrations, involving, in scattered or in diffuse fashion, the interstices and numerous follicles or the entire lobe.* The exudate contained lymphocytes, plasma cells, eosinophils and neutrophils in varying proportions. Within follicles were degenerated epithelial cells, macrophages with or without hemosiderin, and, frequently, disintegrating leukocytes. The colloid was altered in varied fashion; it was liquefied, transformed into small globules or appeared as granular debris. Faint outlines of degenerated cells were common in such follicles. In some instances in the dog and rabbit, moderate fibrosis was observed.

The sections from control animals and from the thyroid glands designated as normal, exhibited the well known placid colloid follicular pattern as a uniform feature. There were neither interstitial inflammatory infiltration nor conspicuous alteration in the appearance of the colloid. Rare desquamated epithelial cells within normal-appearing colloid follicles were not considered to be of pathologic significance.

## Experimental Observations

The results obtained in the entire series of experiments are shown in Tables I to XI. The controls, 30 rabbits and 5 guinea pigs, are listed in Table I. In all of these, the blood was tested for homologous antithyroid antibody. Table I also indicates the results obtained in 6 rabbits and 7 guinea pigs which had been given injections of adrenal extract, and 3 rabbits which had received injections of pituitary extract. Unless otherwise stated, all inoculums used in the controls were introduced intradermally in combination with the complete Freund adjuvants. Tables II to VI and XI represent the data obtained from immunization with autologous or homologous thyroid extract in 15 dogs, 67 rabbits and 27 guinea pigs. Tables VII to X record the results of experiments with heterologous thyroid extract in 41 rabbits.

The control animals clearly revealed the absence of thyroid-specific antibody and the absence of histologic abnormalities in the thyroids.

* This method of grading is not the same used in reference 5.

TABLE I

CONTROLS

| No. of animals used | Inoculum | No. of injections | Method of injection | Duration (mo.) | Antibody titer * | Histologic alteration |
|---|---|---|---|---|---|---|
| *Rabbits* | | | | | | |
| 5 | Pooled homologous serum | 1 | | 3 | 0 | 0 |
| 1 | Normal human serum | 1 | | 1 | 0 | 0 |
| 1 | Normal hog serum | 1 | | 1 | 0 | 0 |
| 3 | Pooled rabbit testicular extract | 2 | | 1 | 0 | 0 |
| 8 | Pooled rabbit pancreatic extract | 2 | | 5 to 7 | 0 | 0 |
| 5 | Complete adjuvants with buffered saline | 1 | | 3 | 0 | 0 |
| 3 | Complete adjuvants without buffered saline | 3 | | 3 | 0 | 0 |
| 2 | Homologous thyroid extract without adjuvants | 2<br>1 | i.d.<br>i.v. | 2 | 0 | 0 |
| 1 | Homologous thyroid extract without adjuvants | 2<br>9 | i.d.<br>i.v. | 10 | 0 | 0 |
| 1 | Human pancreatic cancer extract | multiple | i.v. | 7 | 0 | 0 |
| *Guinea pigs* | | | | | | |
| 5 | Homologous pancreatic extract | | | 4 | 0 | 0 |
| | | *Additional controls* | | | | |
| *Rabbits* | | | | | | |
| 2 | Autologous adrenal extract | | | 1 and 2 | | 0 |
| 2 | Homologous adrenal extract | | | 1 and 2 | | 0 |
| 1 | Autologous and homologous adrenal extract | | | 1 | | 0 |
| 1 | Human adrenal extract | | | 1 | | 0 |
| 3 | Homologous pituitary extract | | | 2 to 2.5 | | 0 |
| *Guinea pigs* | | | | | | |
| 6 | Homologous adrenal extract | | | 2 to 4 | | 0 |
| 1 | Beef adrenal extract | | | 1 | | 0 |

i.d.—intradermal.
i.v.—intravenous.

* Tested against homologous (rabbit or guinea pig) thyroid extract.

Pooled rabbit, human, and hog serums, extracts of homologous testis, pancreas, adrenal, pituitary, and of human pancreatic cancer failed to alter the thyroid structure in any way. The complete Freund adjuvants used without the addition of any extract gave negative results. This was also the case with pooled homologous thyroid extract, when injected

TABLE II

EFFECT OF AUTOLOGOUS AND HOMOLOGOUS THYROID EXTRACT IN THE DOG

| Dog no. | Inoculum | No. of injections | Duration (mo.) | Antibody titer | Histologic alteration |
|---|---|---|---|---|---|
| 1184 | PTE | 3 | 8 | 0 | ++ |
| 1186 | ATE | 1 | 1* | 0 | 0 |
| 1196 | ATE | 1 | 8 | 0 | ++ |
| | PTE | 3 | | | |
| 1198 | ATE | 1 | 8 | 0 | 0 |
| | PTE | 3 | | | |
| 1514 | PTE | 8 | 29 | 20 | ++ |
| 1600 | PTE | 8 | 29 | 80 | ++ |
| 1609 | PTE | 8 | 29 | 0 | + |
| 1614 | PTE | 8 | 29 | 80 | ++ |
| 1620 | PTE | 1 | 1* | 0 | 0 |
| 1622 | PTE | 1 | 1* | 0 | 0 |
| 1519 | PTE | 1 | 1* | not examined | + |
| 1191 | † | 0 | 8 | 0 | 0 |
| 1623 | PTE | 1 | 3 | 0 | (right lobe) + |
| | PTE | 8 | 26 | 320 | (left lobe) ++ |
| 1521 | PTE | 4 | 11 | 0 | (left lobe) + |
| | PTE | 6 | 22 | 320 | (right lobe) + |
| 1618 | PTE | 5 | 16 | 320 | (left lobe) ++ |
| | PTE | 8 | 24 | 320 | (right lobe) ++ |

PTE = pooled thyroid extract.
ATE = autologous thyroid extract
* Died
† Hemithyroidectomy, no injections given.

without the addition of the Freund adjuvants. Moreover, in 12 guinea pigs, homologous extract of pancreas or adrenal and heterologous adrenal extract had no effect on the thyroid.

### *Dog Investigations*

Table II represents the effects of active immunization of 15 dogs with homologous thyroid extract. In 10 there was slight or marked thyroiditis. Severe lesions were produced in the glands of two dogs (nos. 1184 and 1196) by pooled dog thyroid extract alone and by a combination of autologous and homologous extract (Figs. 1 and 2). Figures 3 and 4 clearly show the difference in the histologic alteration in the right and

left lobes of the same dog (no. 1623), after 3 and 26 months, following 1 and 8 injections of homologous extract, respectively.

Four dogs died 4 weeks after a single injection of pooled homologous thyroid extract. No antibody was detected in the blood of 3 of these, and their thyroid glands were normal. In the thyroid of the fourth dog, slight histologic alterations were present. These were comparable to those shown in Figure 3. The blood of this dog was not tested for antibodies.

The conclusions drawn from these experiments are as follows:

Slight alterations were observed in the thyroids of dogs immunized with pooled homologous extract over a period of 1 to 11 months. These changes, however, were not accompanied by the appearance of detectable antibody in the blood. Repeated supplementary inoculations (5 to 8) caused antibodies to appear 16 to 26 months after the first injection. In a single dog there was a change from slight thyroiditis 3 months after a single injection, to a severe thyroiditis after 7 supplementary injections in the course of 23 months. The change in the lesion was paralleled by a rise of antibody titer from 0 to 320. Another dog, in which the left and right lobes were examined after an interval of 11 and 22 months, respectively, exhibited no significant change in the degree of thyroiditis. This, despite the fact that antibody was absent after 11 months and appeared in a titer of 320 at 22 months; there had been 2 supplementary inoculations in the interval. In a third dog, both titer levels were the same, and the lesions in left and right lobes were very similar after 5 and 8 injections and a lapse of 16 and 26 months, respectively. On the other hand, unusually severe thyroid lesions after repeated injections with pooled thyroid extract in 3 dogs were at no time accompanied by a demonstrable antibody titer when tested after 8 and 29 months. Hemithyroidectomy alone, not followed by injection of extract, resulted in neither the production of thyroid-specific antibodies nor of histologic lesions in the remaining lobe after a period of 8 months.

Cross circulation experiments in 5 additional dogs (not listed in Table II) which received donor blood from 5 dogs with antithyroid antibody titers of 80 to 160, also gave negative results. In the recipient dogs, the transferred antibody disappeared rapidly, and the thyroids, when examined 2 to 6 months later, revealed no alterations.

### *Rabbit Investigations*

Table III represents the results of a single injection of autologous and homologous rabbit thyroid extract. In the absence of antibody, there were no alterations of thyroid structure in two animals. Distinct histologic lesions were observed at relatively low titers (640) as well as at 10 times higher levels, regardless of whether the animals were sacrificed

at 2 or at 5 months. In the presence of antibody, moderate to severe thyroiditis occurred within 2 months of a single injection of autologous or homologous thyroid extract (Figs. 7 and 8). The minor lesions in the

TABLE III

EFFECT OF AUTOLOGOUS AND HOMOLOGOUS THYROID EXTRACT IN THE RABBIT

| Rabbit no. | Inoculum | No. of injections | Duration (mo.) | Antibody titer | Histologic alteration |
|---|---|---|---|---|---|
| 202 | ATE | 1 | 3 | 1250 | o |
| 203 | ATE | 1 | 3 | 640 | ++ |
| 205 | ATE | 1 | 3 | 1250 | + |
| 206 | ATE | 1 | 3 | 640 | ++ |
| 207 | ATE | 1 | 3 | o | o |
| 209 | ATE | 1 | 3 | o | o |
| 210 | ATE | 1 | 5 | 1250 | + |
| 212 | ATE | 1 | 5 | 640 | o |
| 214 | PTE | 1 | 4.5 | 640 | ++ |
| 215 | PTE | 1 | 2.5 | 6250 | ++ |
| 216 | PTE | 1 | 2 | 640 | ++ |
| 217 | PTE | 1 | 5 | 6250 | ++ |
| 218 | PTE | 1 | 4.5 | 6250 | ++ |
| 219 | PTE | 1 | 2.5 | 6250 | ++ |
| 220 | PTE | 1 | 2.5 | 6250 | ++ |
| 221 | PTE | 1 | 2.5 | 6250 | ++ |
| 223 | PTE | 1 | 2.5 | 640 | ++ |
| 224 | PTE | 1 | 2.5 | 1250 | o |

ATE = autologous thyroid extract
PTE = pooled thyroid extract

thyroid of 2 rabbits with intermediate titer ranges were of the same intensity after 3 and 5 months. In 3 animals, however, despite the presence of circulating antibody, the microscopic pattern was that of normal thyroid.

In Table IV the effects of repeated injections (2 to 4) with homologous thyroid extract are listed. In this series of experiments, severe histologic lesions were more regularly observed in association with higher antibody titers (320 to 3125). Figures 5, 6, 9 and 10 are representative examples of thyroid alterations encountered in this group. Minor lesions were accompanied by lower levels of antibody (16 to 160). In 3 animals without circulating antibody, the thyroid glands were essentially normal; one rabbit had evidence of mild thyroiditis only. On the other hand, the thyroid was normal in 4 rabbits with both low and high antibody titers. The time factor and the number of injections seemed to have no influence on titer level or on the extent of histologic alteration. Slight and marked lesions were of the same intensity after 2, 3 or 4 injections and after intervals of 3½, 7 and 13 months.

When purified rabbit thyroglobulin was used as the immunizing agent,

TABLE IV

EFFECT OF REPEATED INJECTIONS WITH HOMOLOGOUS THYROID EXTRACT IN THE RABBIT

| Rabbit no. | Inoculum | No. of injections * | Duration (mo.) | Antibody titer | Histologic alteration |
|---|---|---|---|---|---|
| 288 | PTE | 2 | 3.5 | 80 | + |
| 289 | PTE | 2 | 3.5 | 640 | ++ |
| 290 | PTE | 2 | 3.5 | 625 | ++ |
| 292 | PTE | 2 | 3.5 | 125 | 0 |
| 293 | PTE | 2 | 3.5 | 0 | 0 |
| 294 | PTE | 2 | 3.5 | 3125 | ++ |
| 297 | PTE | 2 | 2.5 | 40 | 0 |
| 298 | PTE | 2 | 3.5 | 3125 | 0 |
| 299 | PTE | 2 | 3.5 | 3125 | ++ |
| 401 | PTE | 2 | 3.5 | 3125 | 0 |
| 402 | PTE | 2 | 3.5 | 1250 | ++ |
| 555 | PTE | 4 | 13 | 80 | + |
| 556 | PTE | 4 | 13 | 16 | +† |
| 557 | PTE | 4 | 13 | 0 | 0 |
| 558 | PTE | 3 | 7 | 1280 | ++ |
| 559 | PTE | 3 | 13 | 160 | + |
| 560 | PTE | 3 | 13 | 320 | ++† |
| 561 | PTE | 3 | 13 | 0 | 0 |
| 562 | PTE | 2 | 5 | 0 | + |
| 563 | PTE | 2 | 13 | 40 | + |

PTE = pooled thyroid extract.

* All injections given at 2-month intervals.

† Cytoplasmic swelling.

TABLE V

EFFECT OF PURIFIED HOMOLOGOUS RABBIT THYROGLOBULIN

| Rabbit no. | Inoculum | No. of injections | Duration (mo.) | Antibody titer | Histologic alteration |
|---|---|---|---|---|---|
| 417 | PRTG | 1 | 3 | 2560 | ++ |
| 418 | PRTG | 1 | 3 | 729 | ++ |
| 419 | PRTG | 1 | 2 | 2560 | ++ |
| 420 | PRTG | 1 | 2.5 | 243 | ++ |
| 421 | PRTG | 1 | 2.5 | 2187 | + |
| 422 | PRTG | 1 | 2.5 | 6561 | ++ |

PRTG = purified rabbit thyroglobulin.

(Table V) the result was uniformly positive in all 6 rabbits tested. The titer of antibody, however, was not the determining factor in respect to the severity of thyroiditis. The histologic alterations were as severe at

TABLE VI

EFFECT OF A SINGLE INJECTION WITH AUTOLOGOUS EXTRACT FOLLOWED BY ONE OR TWO INJECTIONS WITH HOMOLOGOUS EXTRACT

| Rabbit no. | No. of PTE injections * | Duration (mo.) | Antibody titer | Histologic alteration |
|---|---|---|---|---|
| 464 | 2 | 9 | 160 | ++ |
| 465 | 1 | 6 | 40 | ++ |
| 467 | 2 | 9 | 45 | o |
| 468 | 2 | 9 | 135 | ++ |
| 469 | 1 | 6 | 405 | ++ |
| 471 | 1 | 6 | 1215 | ++ |
| 472 | 1 | 6 | 405 | ++ |
| 473 | 2 | 9 | 405 | + |
| 485 | 1 | 3.5 | 125 | + |
| 486 | 1 | 3.5 | 625 | + |
| 487 | 1 | 3.5 | 625 | + |
| 488 | 1 | 3.5 | 125 | ++ |
| 489 | 1 | 3.5 | 625 | + |
| 475 | 1† | 3.5 | o | o |
| 476 | 1 | 3.5 | o | o |
| 477 | 1 | 3.5 | 25 | + |
| 478 | 1 | 3.5 | 25 | o |
| 479 | 1 | 3.5 | o | o |
| 480 | 1 | 3.5 | 25 | + |
| 481 | 1 | 3.5 | o | + |
| 482 | 1 | 3.5 | 125 | ++ |
| 483 | 1 | 3.5 | 25 | + |
| 484 | 1 | 3.5 | o | o |

* Injections of PTE (pooled thyroid extract) were given after a single injection of autologous thyroid extract.

† Tubercle bacilli omitted from Freund adjuvants in injections to rabbits 475 to 484.

low titers of antibody (243) as at titers of 20 times as high, and were less marked at an intermediate titer range.

Table VI exhibits the combined effect of autologous and homologous thyroid extract. In the experiments listed in the lower half of the table, tubercle bacilli were omitted from the Freund adjuvants. In the animals sacrificed after 3½, 6 and 9 months, the intensity of thyroiditis did not differ significantly in relation to the time factor. Nor was there a correla-

tion of the titer levels with respect to the duration of the experiments or the degree of thyroiditis. In fact, the only animal in this series with a histologically normal thyroid had an antibody titer as low (45) as that in one with severe thyroiditis.

When the tubercle bacilli were omitted from the adjuvant, the histologic alterations were only slight and the titers were low (25), except in one animal in which a higher titer (125) was accompanied by distinct histologic lesions. In 4 of 10 animals, there were neither circulating antibodies nor evidences of histologic abnormality. While in this group the antibody levels were considerably below those observed in the rabbits listed in Tables III and V, the omission of the tubercle bacilli from the adjuvant might have caused the depressant effect on antibody formation. The omission resulted in less marked or absent histologic alterations in the thyroid.

The data recorded in Tables III to VI may be summarized briefly as follows:

Fifty-seven rabbits received injections of rabbit thyroid extract or purified rabbit thyroglobulin, in each instance combined with the complete Freund adjuvants. Thyroiditis occurred in 45, more than three fourths of the total. The histologic alterations correlated with positive antibody titers in 41 and the existence of normal thyroid with negative titers in 5; a parallelism thus was found in 80.7 per cent of the animals. In 8 animals with elevated titers, there was no histologic alteration. The correlation of thyroiditis with the presence of antibody in the blood, however, was not quantitative. Slight or severe histologic alterations did not regularly correlate with low or high antibody titers. Nor did the degree of thyroiditis have any relation to the duration of the experiment (time factor). It was concluded that the thyroiditis brought about by immunization with autologous and homologous thyroid extract was not necessarily progressive, even in the presence of a high circulating antibody titer.

The results of immunization of rabbits with heterologous extract, prepared from hog, dog, beef and human thyroid tissue, are listed in Tables VII to X.

Eight rabbits received a single intradermal injection with hog thyroid extract and were sacrificed after 1½ to 8½ months. In only one of these, sacrificed after 8 months, was there a slight thyroiditis; this was accompanied by a relatively low antibody titer (80). In the remaining 7 rabbits, the thyroid was normal histologically although the antibody titer ranged between 40 and 135 in animals sacrificed after 2 months and between 20 and 80 in animals sacrificed after 3½ to 8½ months.

Following repeated intravenous injections of purified hog thyro-

## TABLE VII

EFFECT OF HETEROLOGOUS HOG THYROID EXTRACT, PURIFIED HOG THYROGLOBULIN AND THYRO-ALBUMIN

| Rabbit no. | Inoculum | No. of injections | Method of injection | Duration (mo.) | Antibody titer * | Histologic alteration |
|---|---|---|---|---|---|---|
| 423 | HTE | 1 | | 2 | 45 | o |
| 424 | HTE | 1 | | 2 | 135 | o |
| 522 | HTE | 1 | | 8.5 | 80 | + |
| 523 | HTE | 1 | | 8.5 | 20 | o |
| 524 | HTE | 1 | | 3.5 | 40 | o |
| 525 | HTE | 1 | | 3.5 | 20 | o |
| 526 | HTE | 1 | | 1.5 | 40 | o |
| 527 | HTE | 1 | | 1.5 | 40 | o |
| 674 | PHTG | 1 | | 1 | 243 | o† |
| 677 | PHTG | 1 | | 1 | 243 | + |
| 613 | PHTG | multiple | i.v. | 2.5 | 19683 | +† |
| 614 | PHTG | multiple | i.v. | 3 | 19683 | + |
| 676 | PHTG | 1<br>multiple | i.d.<br>i.v. | 5 | 729 | +† |
| 678 | PHTG | 1<br>multiple | i.d.<br>i.v. | 5 | 2187 | ++ |
| 642 | PHTA | 1<br>multiple | i.d.<br>i.v. | 3 | o | o |
| 643 | PHTA | 1<br>multiple | i.d.<br>i.v. | 3 | o | o |

HTE = hog thyroid extract.
PHTG = purified hog thyroglobulin.
PHTA = purified hog thyralbumin.
i.v. = intravenous.
i.d. = intradermal.

* Tested against rabbit thyroid.
† Cytoplasmic swelling.

## TABLE VIII

EFFECT OF HETEROLOGOUS DOG THYROID EXTRACT IN THE RABBIT

| Rabbit no. | Inoculum | No. of injections | Method of injection | Duration (mo.) | Antibody titer * | Histologic alteration |
|---|---|---|---|---|---|---|
| 546 | CTE | 1 | i.d. | 2 | o | o |
| 663 | CTE | 1 | i.d. | 1 | 729 | o |
| 664 | CTE | 1 | i.d. | 3 | 243 | o |
| 665 | CTE | 1 | i.d. | 1 | 81 | +† |
| 666 | CTE | 1 | i.m. | 1 | o | o† |
| 667 | CTE | 1 | i.m. | 3 | o | o |
| 668 | CTE | 1 | i.m. | 1 | o | +† |

CTE = canine thyroid extract.
i.d. = intradermal.
i.m. = intramuscular.

* Tested against rabbit thyroid.
† Cytoplasmic swelling.

TABLE IX

EFFECT OF HETEROLOGOUS BEEF THYROID EXTRACT IN THE RABBIT

| Rabbit no. | Inoculum | No. of injections | Duration (mo.) | Antibody titer * | Histologic alteration |
|---|---|---|---|---|---|
| 507 | BTE | 1 | 6 | 320 | o |
| 508 | BTE | 1 | 6 | 80 | + |
| 509 | BTE | 1 | 6 | 320 | o |
| 510 | BTE | 1 | 6 | 160 | o |
| 511 | BTE | 1 | 6 | 160 | o |
| 512 | BTE | 1 | 6 | 1280 | + |

BTE = beef thyroid extract.
* Tested against rabbit thyroid.

TABLE X

EFFECT IN THE RABBIT OF HETEROLOGOUS EXTRACT OF NORMAL, COLLOID NODULAR, AND CANCEROUS HUMAN THYROID

| Rabbit no. | Inoculum | No. of injections | Method of injection | Duration (mo.) | Antibody titer * | Histologic alteration |
|---|---|---|---|---|---|---|
| 530 | HTE | 1 | | 2 | o | o |
| 531 | HTE | 1 | | 3.5 | o | o† |
| 532 | HTE | 1 | | 3 | o | o |
| 533 | HTE | 1 | | 3.5 | o | o |
| 536 | HTE | 1 | | 3.5 | 27 | o |
| 537 | HTE | 1 | | 3.5 | 27 | o |
| 540 | HCGE | 1 | | 3 | 81 | o |
| 541 | HCGE | 1 | | 3 | 81 | o |
| 538 | HTCaE | 1<br>1 | i.d.<br>i.p. | 3 | o | o |
| 539 | HTCaE | 1<br>1<br>multiple | i.d.<br>i.p.<br>i.v. | 3 | o | o† |
| 542 | HTCaE | 1<br>1<br>multiple | i.d.<br>i.p.<br>i.v. | 4 | o | o |
| 543 | HTCaE | 1<br>1<br>multiple | i.d.<br>i.p.<br>i.v. | 4 | o | o† |

HTE = human thyroid extract.
HCGE = human colloid goiter extract.
HTCaE = human thyroid cancer extract.
i.d. = intradermal.
i.p. = intraperitoneal.
i.v. = intravenous.

* Tested against rabbit thyroid
† Cytoplasmic swelling.

globulin, a very high anti-rabbit thyroid antibody titer was found in the blood of 2 rabbits when sacrificed after 3 months. Despite this, the histologic alterations were of relatively slight degree. Only one of the animals (no. 678), with an intermediate titer range, had severe thyroiditis. Repeated intravenous injections of hog thyro-albumin gave entirely negative results.

Extracts of dog thyroid were injected intramuscularly into 3 rabbits. In only one of these, sacrificed one month later, was there slight thyroiditis. No anti-rabbit thyroid antibodies appeared in the blood of any of these 3 animals. A single intradermal injection of the same extract gave somewhat similar results. Very slight thyroiditis accompanied relatively low antibody titers. In 2 animals with higher titer levels, there was no evidence of thyroiditis at all.

When beef extract was injected, slight thyroiditis appeared in 2 rabbits. This was accompanied by a very low titer in one animal and by a very high one in the other. The thyroids were normal in the 4 remaining animals with intermediate titer ranges (2 to 4 times the lowest titer).

While the administration of extracts from normal human thyroid and from benign colloid goiter resulted in very low antibody titers against rabbit thyroid, no antibody was detected in 4 rabbits receiving saline extract of cancerous thyroid. In none of the rabbits of this series were there evidences of thyroiditis.

The data listed in Tables VII to X may be summarized as follows:

The injections into rabbits of heterologous extracts prepared from the thyroids of beef, hog, and dog were followed in one or two instances in each group by slight thyroiditis. The lesions were paralleled by low antibody titers (80) when tested against rabbit thyroid, except in one animal with a high titer range (1280). None of the other rabbits immunized with heterologous thyroid extracts, including that procured from human thyroid, exhibited evidence of histologic alteration.

Distinct thyroiditis was present, however, in 5 rabbits, following repeated intravenous injections with purified hog thyroglobulin. These lesions developed in the course of 1 to 5 months. Figure 11 represents the lesion in one of the rabbits; the histologic changes in the remainder were less marked.

In addition to the histologic patterns of thyroiditis thus far described, there was also a peculiar, foamlike swelling of the follicular epithelial cytoplasm. This was observed in some of the rabbit glands after the combined injection of autologous and pooled homologous extracts. The inflammatory lesions in these instances were comparatively slight and appeared unrelated to the epithelial swelling. The foamy alteration was even more marked following injections of dog thyroid extract, after re-

peated intravenous injections of purified hog thyroglobulin and extracts of both normal and cancerous human thyroid tissue (Fig. 13). Since only paraffin sections were available, conventional fat stains on frozen sections were not possible. The Ciaccio stain, however, failed to demonstrate the presence of lipid. The peculiar swelling resembled a process of hydration or storage and was combined in one instance with distinct cytolysis of follicular epithelium (Fig. 14).

### *Guinea Pig Investigations*

Table XI combines the results in 2 groups handled in slightly different manner. In the first, 19 guinea pigs received intradermal injections of pooled homologous thyroid extract. The histologic alterations in the thyroids of these animals were for the most part comparatively slight. In 10 of the 19, there was no detectable antibody against guinea pig thyroid.

The 8 guinea pigs listed in the lower part of the table first received injections into the skin of the neck region, and, 2 months later, into the footpads; in each instance the inoculum was pooled guinea pig thyroid extract.

While these animals uniformly showed significant thyroiditis, there was no quantitative correlation between this and the amount of circulating antibody. An unusually high titer (3125) was accompanied by comparatively minor lesions, and in guinea pigs with severe thyroiditis, the antibody titers were as low as 25 and did not exceed 125.

The lesions in the guinea pigs were similar to those observed in the rabbits. Hemosiderin granules were occasionally seen in the colloid, and clumps of erythrocytes appeared in a few follicles; in a few instances, these were engulfed by macrophages. The interstitial inflammatory reaction and the reduction in size of colloid-bearing follicles were as marked as in the rabbit lesions (Fig. 12).

One of the guinea pigs (Table XI) was pregnant. In this animal, there was only slight focal lymphocytic infiltration in the thyroid and no detectable antibody in the blood. The thyroids of its 3 offspring were normal.

## Discussion

In the dog, rabbit, and guinea pig, each immunized with species-homologous thyroid extract, the histologic lesion of thyroiditis was of a similar pattern. It was characterized by a reduction in the size of colloid-filled follicles and an infiltration by inflammatory cells of varied types. Differences existed not only in the extent of these lesions, especially in the rabbit and guinea pig, but also in the cellular composition of the exudates. The accumulation of lymphocytes and plasma cells and—

especially in the rabbit—eosinophils, was a striking feature. Focal infiltrations were present in all affected glands, even if the bulk of the thyroid tissue was not significantly altered. The lumens of the follicles,

TABLE XI

EFFECT OF HOMOLOGOUS THYROID EXTRACT IN THE GUINEA PIG

| Guinea pig no. | Inoculum | No. of injections | Duration (mo.) | Antibody titer | Histologic alteration |
|---|---|---|---|---|---|
| 1 | PGTE | 2* | 7.5 | 20 | + |
| 2 | PGTE | 2 | 7.5 | 40 | ++ |
| 3 | PGTE | 2 | 7.5 | 80 | + |
| 4 | PGTE | 2 | 7.5 | 40 | + |
| 5 | PGTE | 2 | 7.5 | 160 | + |
| 6 | PGTE | 1 | 7.5 | 0 | + |
| 7 | PGTE | 1 | 7.5 | 80 | + |
| 8 | PGTE | 1 | 7.5 | 0 | + |
| 9 | PGTE | 1 | 7.5 | 160† | + |
| 10 | PGTE | 1 | 7.5 | 0 | + |
| 11 | PGTE | 1 | 7.5 | 0 | + |
| 12 | PGTE | 1 | 7.5 | 80 | + |
| 13 | PGTE | 2 | 7.5 | 0 | + |
| 14 | PGTE | 2 | 7.5 | 0 | + |
| 16 | PGTE | 2 | 7.5 | 0 | + |
| 17 | PGTE | 2 | 7.5 | 81 | + |
| 18 | PGTE | 1 | 7.5 | 0 | + |
| 19 | PGTE | 1 | 7.5 | 0 | + |
| 20 | PGTE | 1 | 9.5‡ | 0 | + |
| 78 | PGTE | 2 | 3.5 | 3125 | + |
| 80 | PGTE | 2 | 3.5 | 25 | ++ |
| 81 | PGTE | 1 | 2.5 | 25 | + |
| 83 | PGTE | 1 | 2.5 | 25 | ++ |
| 86 | PGTE | 2 | 5.5 | 125 | ++ |
| 87 | PGTE | 2 | 5.5 | 125 | + |
| 88 | PGTE | 2 | 2.5 | 125 | ++ |
| 90 | PGTE | 2 | 2.5 | 25 | ++ |

PGTE = pooled guinea pig thyroid extract.

* Where a second injection was given, it was after a 2 month interval.

† Positive skin test.

‡ Pregnant; 3 offspring revealed no histologic alteration.

adjacent to denser infiltrations, contained various cells: macrophages (viable or in the process of disintegration), eosinophils (especially in the rabbit), neutrophils (particularly in the dog and rabbit), and

lymphocytes in all 3 species. The homogeneity of the colloid disappeared and was replaced in part by globular particles or granular debris. The follicular epithelium showed vacuolar degeneration and desquamation. Disintegration of various cells within the follicles was a prominent feature. Disruption of the follicle wall was occasionally seen, especially in the rabbit and guinea pig, and was accompanied by cytolytic alterations in the epithelium. Regenerative activity was suggested by a few solid tubules of thyroid epithelium containing mitotic figures. Focal fibrosis was also encountered in a few instances, especially in the dog and rabbit. The occasional presence of hemosiderin-containing cells in the densely inflamed stroma or within intact thyroid follicles clearly pointed to extravasation of erythrocytes. The site of inoculation was such that there was no trauma to the thyroid in these animals.

The thyroiditis was brought about experimentally in these animals by one or repeated intradermal injections of homologous thyroid extract. In the initiation of the thyroiditis, an interaction between a thyroid-specific antibody and its selective target, the colloid-bearing follicles, was the foremost pathogenetic factor. No lesions were encountered in any other organ examined in these animals, except for a slight or moderate degree of interstitial pyelonephritis in 11 guinea pigs and in 2 rabbits. These lesions, we feel, were not related to the immunization procedure. Tissues examined were parathyroid, adrenal, heart, spleen, lung, liver, kidney, pancreas, testis, ovary and, in several dogs and rabbits, the pituitary.

The failure of several rabbits to exhibit evidence of thyroiditis, despite the presence of specific thyroid antibodies in the blood (Tables III and IV), has not been satisfactorily explained. Perhaps the paucity of thyroid tissue available for histologic examination in this group might have introduced an artificial factor of technical nature. In some of the animals, only a few sections were available. Small focal inflammatory infiltrations might have been present in portions of the thyroid not examined; considerable tissue was used in testing for thyroid antigen content. In two rabbits, in which the initial preparations were negative, additional sections were procured, and these revealed focal inflammatory lesions of moderate severity.

In two dogs (Table II), with marked thyroiditis, no circulating antibody was detected. It might be assumed that the antibody had disappeared when these dogs were sacrificed. Whether or not the destruction of normal thyroid parenchyma was responsible for its disappearance cannot be determined. The two animals were not tested until shortly before they were sacrificed.

There were 10 guinea pigs (upper part of Table XI) with slight

thyroiditis and without detectable antibody. In general, the antibody titers against guinea pig thyroid were low in this series, ranging between 20 and 80.

That at least part of the thyroid-specific antibody can combine with the intrafollicular colloid is suggested by the positive labeling with fluorescent antibody in frozen sections of normal rabbit thyroid previously acted upon by rabbit auto-antiserum to thyroid. These data, which have been published recently,[10] include the serums from two rabbits listed in Table IV. The histologic lesion in one of these rabbits is illustrated in Figure 10. The presence of macrophages and lymphocytes in a distended lymphatic is of particular interest here since it might represent the response to colloid resorption. Recently, under similar experimental conditions, we have observed well stained colloid surrounded by numerous lymphocytes within a lymph vessel of the thyroid. This rabbit had received 3 injections of pooled rabbit thyroid extract and was sacrificed after 3 months. There was moderately severe focal thyroiditis in the periphery of the gland close to the colloid-containing lymphatic; in this instance the titer of auto-antibody was 7,290.

Various attempts to produce thyroiditis in experimental animals by vitamin deficiencies and by the administration of thiouracil and radioactive iodine have been summarized by Hellwig and Wilkinson.[11] Using irradiated iodine, these authors succeeded in confirming the experiments of Goldberg, Chaikoff, Lindsay and Feller,[12] Gorbman,[13] and of Maloof, Dobyns and Vickery,[14] who produced thyroiditis in white rats and guinea pigs by injecting 1 mc. of $I^{131}$ intraperitoneally. In the course of 4 weeks, the thyroid glands in these animals were replaced by fibrous tissue. The intermediate phases observed by Hellwig and Wilkinson after 2, 4 and 7 days bore some resemblance to the lesions observed in our animals, although the latter had developed in the course of several months following injection of thyroid extract. Hellwig and Wilkinson emphasized that disruption of the follicle wall due to epithelial degeneration resulted in the exposure of the mesenchymal stroma to colloid. Kracht[15] induced a diffuse lymphocytic thyroiditis within 7 days by injecting an ether soluble extract of human thyroid colloid directly into the thyroid of guinea pigs. The lipid protein complexes contained within the extract were considered the agents responsible for the extensive inflammation and foreign body granulomas.

Whatever factors might affect the normal equilibrium between secretion and resorption of colloid, any damage to the lining epithelium or to the follicle content could disturb this balance. Alterations in the colloid itself and in the cytoplasm of follicle epithelium, effected in our experiments by intradermal injection of autologous and homologous thyroid

extract, appeared to be an organ-specific immune response. In a few instances, even heterologous thyroid extract has acted in a similar manner. Various factors, such as the intensity of inflammatory reaction, hemorrhage, the action of macrophages in the resorption of blood and altered colloid, and the regenerative capacity of affected thyroid follicles, apparently contribute to the eventual outcome of the immune response. Variations in the degree of follicle damage and the resulting inflammation are probably responsible—under controlled experimental conditions —for the persistence or disappearance of the circulating thyroid-specific antibody.

From the manner in which these experiments have been carried out, it would be somewhat speculative to postulate a regular sequence in the various histologic alterations. Individual differences in reactability of the animals, moreover, did not permit strict correlation of the structural alterations with the duration of the experiment. Nor was the severity of the lesions necessarily dependent upon the level of the specific antithyroid antibody in the blood of the immunized animals.

## Summary

Thyroiditis has been induced in rabbits, guinea pigs, and dogs by one or more intradermal injections of homologous saline thyroid extract, purified thyroglobulin and, in a few instances, in rabbits only, by heterologous thyroid extract. In all 3 species, the histologic lesions were of a similar type, characterized by a reduction in size and colloid content of the thyroid follicles and by inflammatory reactions indicative of thyroiditis.

The correlation between the intensity of thyroiditis and the level of circulating thyroid-specific antibody titer in the immunized animals was, in general, qualitative rather than quantitative. Correlation was greatest in the rabbit (about 80 per cent), especially when homologous extract alone, or combined with autologous extract, was used.

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[*Illustrations follow*]

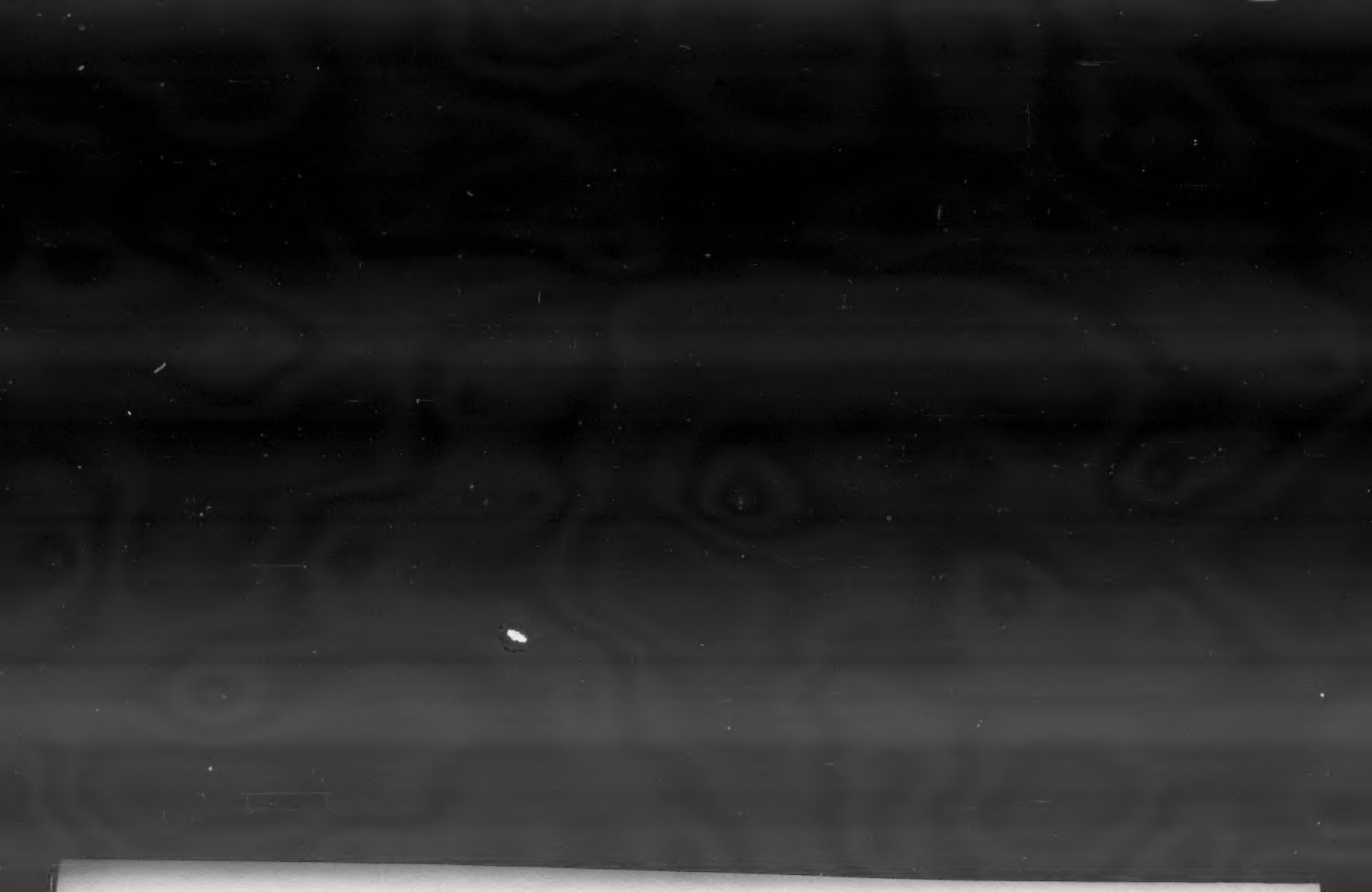
## Legends for Figures

Except where indicated, sections illustrated were stained with hematoxylin and eosin.

Fig. 1. The remaining lobe of a dog (no. 1169) which had first received extract prepared from its own thyroid (hemithyroidectomy) and, later, pooled dog thyroid extract. The animal was sacrificed after 8 months. The normal follicular pattern is obscured by a dense inflammatory reaction. The preserved thyroid follicles contain liquefied colloid infiltrated by leukocytes and macrophages. Other follicles are completely masked by infiltration of neutrophils, lymphocytes, plasma cells, and macrophages. × 200.

Fig. 2. Dog (no. 1184) injected 3 times with pooled dog thyroid extract and sacrificed after 8 months. Small and nearly solid follicles, with little colloid, constitute a striking feature. The diffuse inflammatory exudate contains many leukocytes. × 96.

Figs. 3 and 4. Right and left lobes of dog (no. 1623) listed in Table II. The right lobe was removed after 3 months, following a single injection of pooled dog thyroid extract; the left lobe, after 26 months. Figure 3 shows a normal colloid pattern with slight interstitial inflammation in the center and desquamated cells within adjacent colloid follicles. This was the only lesion in the lobe. Distention and hyperemia of two veins, at the lower left and in the right upper corner, are caused by ligation, incident to the removal of the lobe. In Figure 4, the inflammatory exudate is diffuse. Dense cellular exudate appears in the interfollicular stroma and within numerous follicles. There is marked disintegration of leukocytic nuclei within the follicles. The entire lobe was involved uniformly. × 200.



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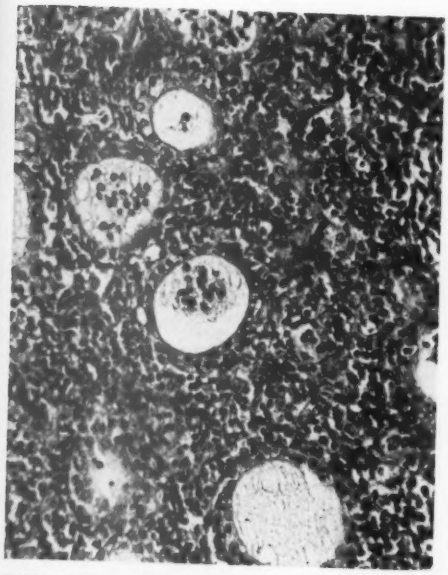

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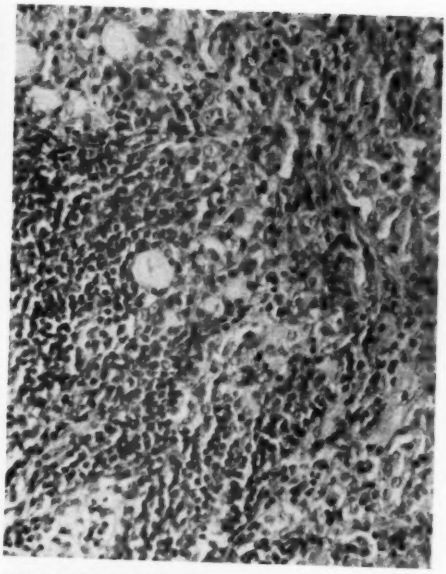

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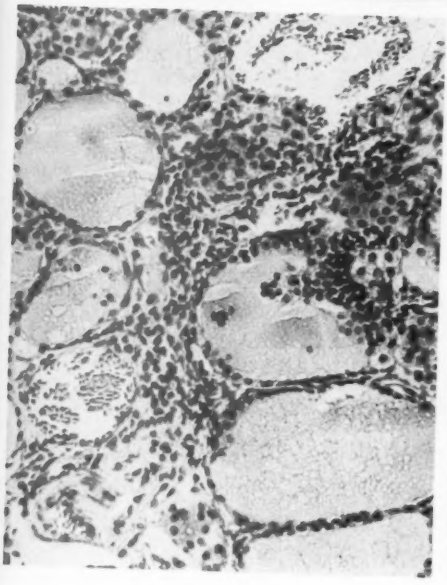

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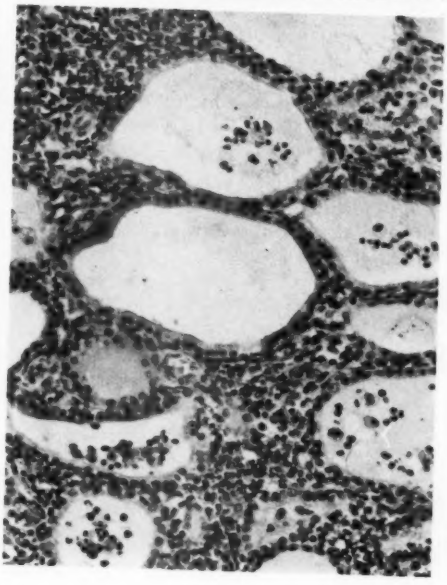

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FIG. 5. Rabbit (no. 402) received 2 intradermal injections of pooled rabbit thyroid extract, 2 months apart, and was sacrificed after 3½ months. Antibody titer against rabbit thyroid, 1:1250. Numerous plasma cells and eosinophils infiltrate the interstitial tissue. The thyroid follicles are of small size. There are many disintegrating leukocytes in the more or less masked follicle lumens. × 200.

FIG. 6. Rabbit (no. 200). The experimental procedure as in rabbit no. 402 (Fig. 5). Titer, 1:625. Interstitial tissue is infiltrated by leukocytes and a few plasma cells in the lower field to the right. The single macrophage in the follicle to the left contains hemosiderin granules. Note also the vacuolated appearance of the colloid and scattered macrophages and leukocytes in several follicles. × 200.

FIGS. 7 and 8. Rabbit (no. 214) received a single intradermal injection of pooled rabbit thyroid extract and was sacrificed after 4½ months. Titer, 1:640. Figure 7 clearly shows the disruption of the follicle wall, detached epithelium and dense infiltrations with lymphocytes and macrophages. In Figure 8 the interstitial infiltration consists of scattered lymphocytes and neutrophils. There are numerous disintegrating cells in the follicles; these represent ghost-like remnants of desquamated cells in the faintly staining colloid. × 200.

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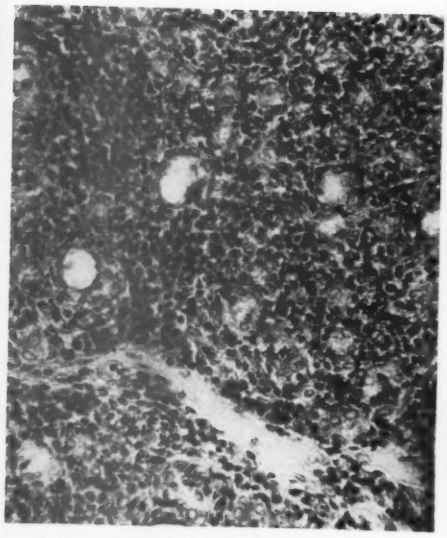

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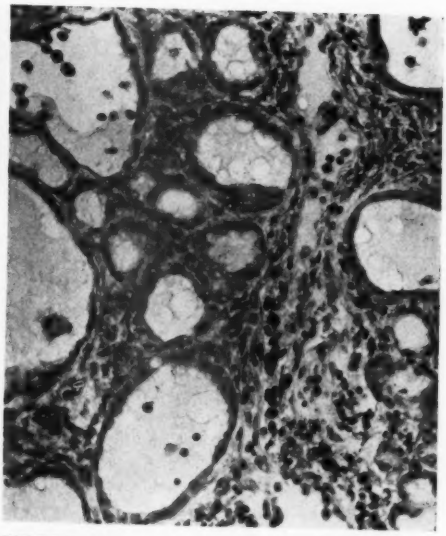

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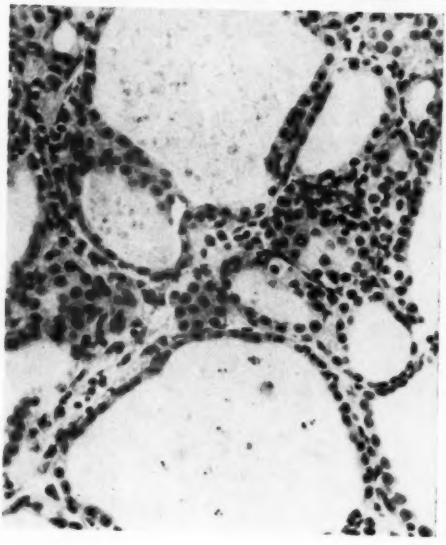

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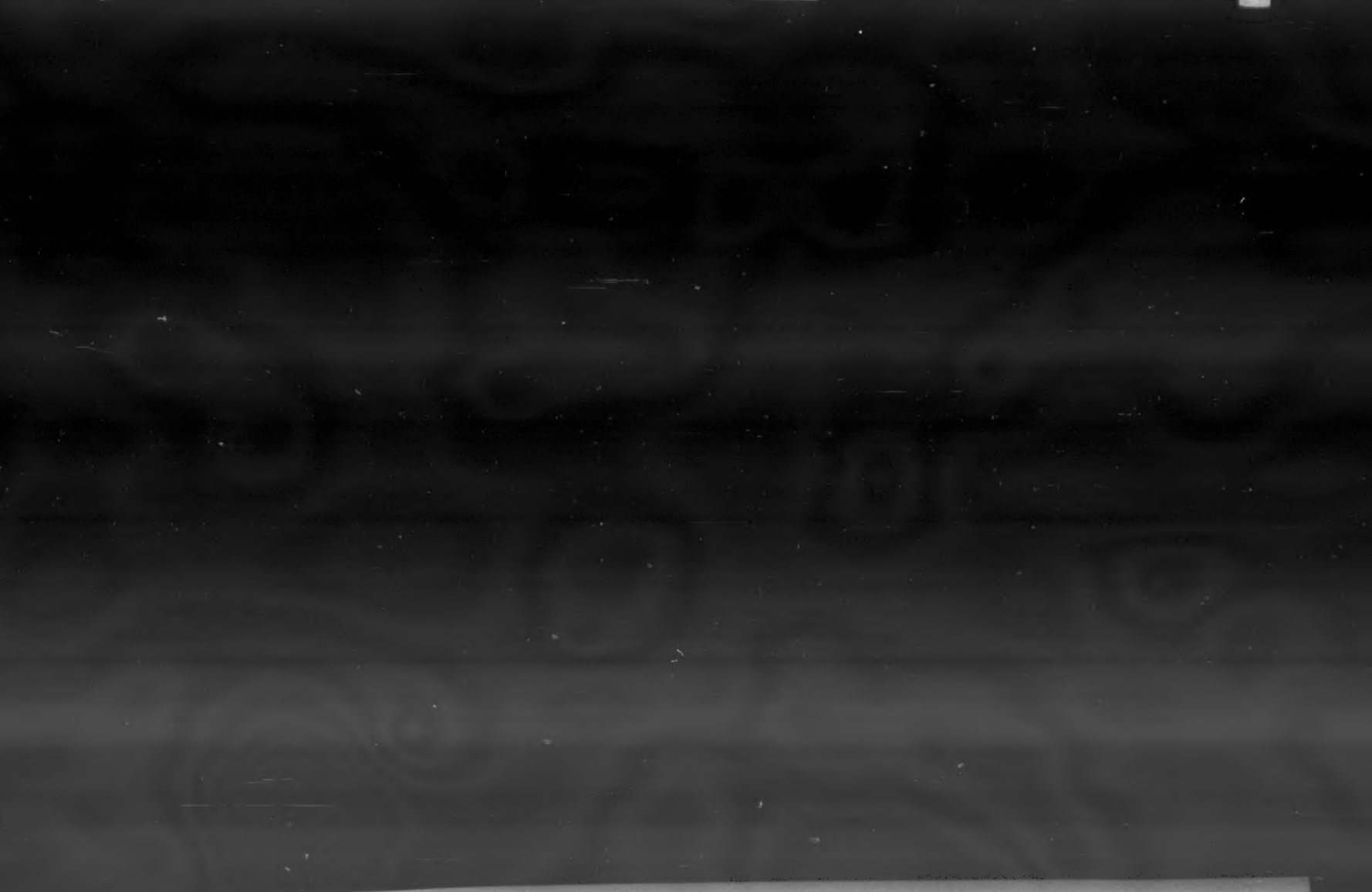
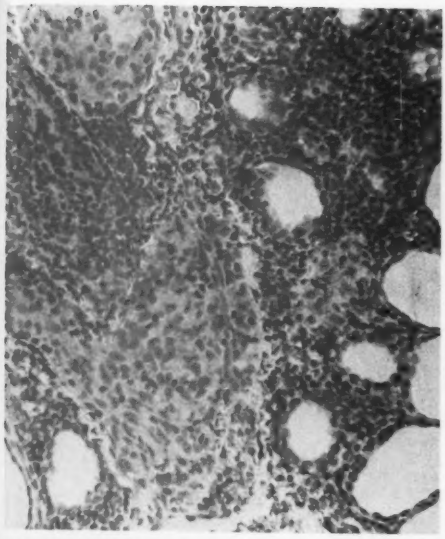
FIG. 9. Rabbit (no. 294) received 2 injections of pooled rabbit thyroid extract and was sacrificed after 3½ months. Antibody titer, 1:325. Note the very dense inflammatory infiltration and proliferating epithelial cells forming solid tubules or nodules. × 96.

FIG. 10. Rabbit (no. 558) received 3 intradermal injections of pooled rabbit thyroid extract and was sacrificed after 7 months. Titer, 1:1280. Interstitial inflammatory infiltration by many eosinophils and some plasma cells. Slight fibrosis may be seen. A distended lymphatic, to the right, contains macrophages and lymphoid cells. × 200.

FIG. 11. Rabbit (no. 678) received one intradermal and numerous intravenous injections of purified hog thyroglobulin and was sacrificed after 5 months. Titer against rabbit thyroid, 1:2187. Several follicles are filled with desquamated epithelial cells, macrophages and leukocytes. There is interstitial infiltration between the preserved follicles which contain unaltered colloid. × 240.

FIG. 12. Guinea pig (no. 88) first received an injection of pooled guinea pig thyroid in the neck region and, 2 months later, in the footpads, and was sacrificed after 2½ months. Titer, 1:125. An area of dense inflammation about small follicles is shown. Several disintegrating cells may be seen in adjacent follicles. × 180.





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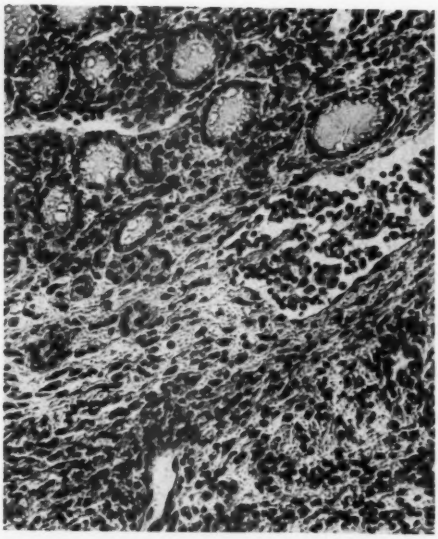

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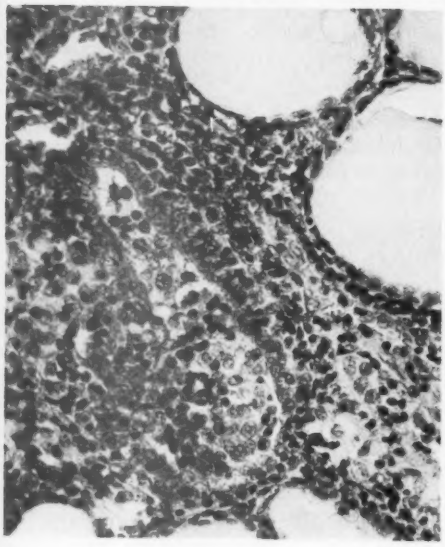

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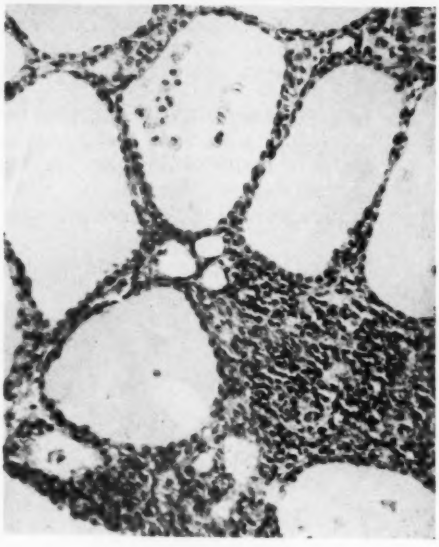

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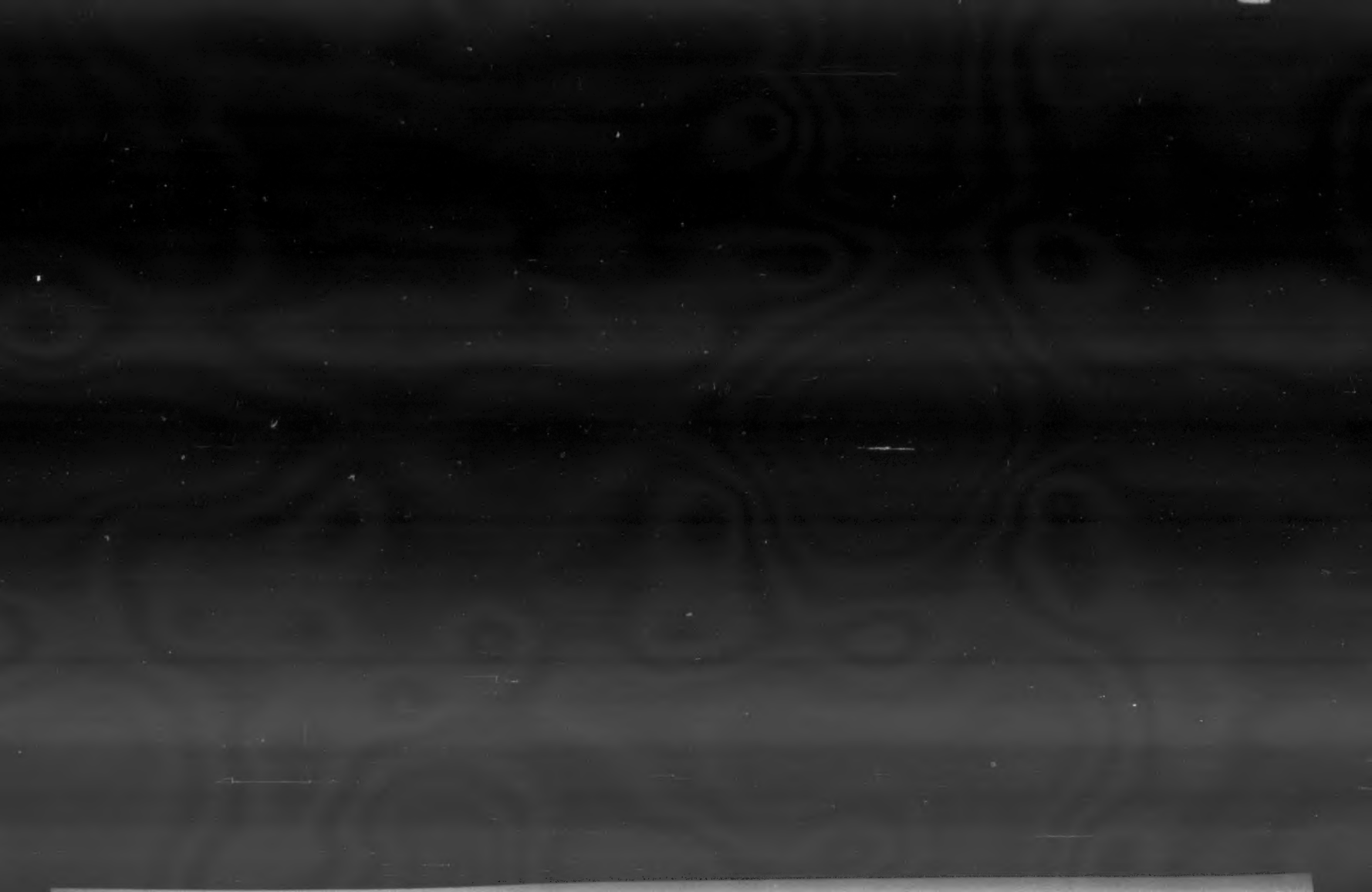
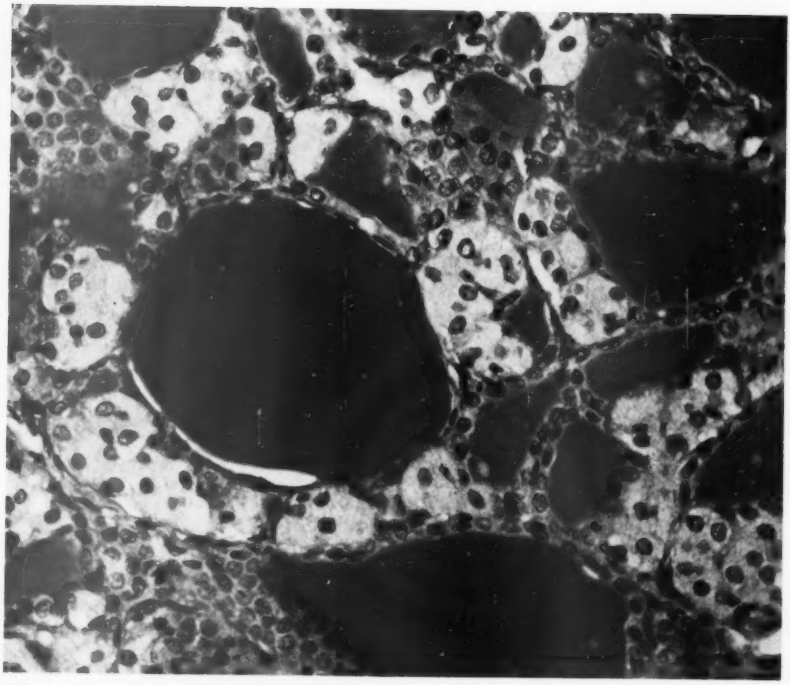
FIG. 13. Rabbit (no. 543) received an extract of human thyroid cancer. A single intradermal and intraperitoneal injection was followed by several intravenous injections. The animal was sacrificed after 4 months. Distinct epithelial swelling with foam cell quality occurs in compact follicles with little or no colloid. Some of the original epithelial cells are undermined and replaced in part by the swollen epithelium. Hotchkiss-McManus stain. × 280.

FIG. 14. Rabbit (no. 539). Material and method of injection as in rabbit no. 543 (Fig. 13). Sacrificed after 3 months. The foamy change is localized within and about the wall of a follicle to the right of the center. Note the disintegration of the epithelial lining in some of the surrounding follicles, with considerable granular and vacuolar debris in their lumens. × 270.





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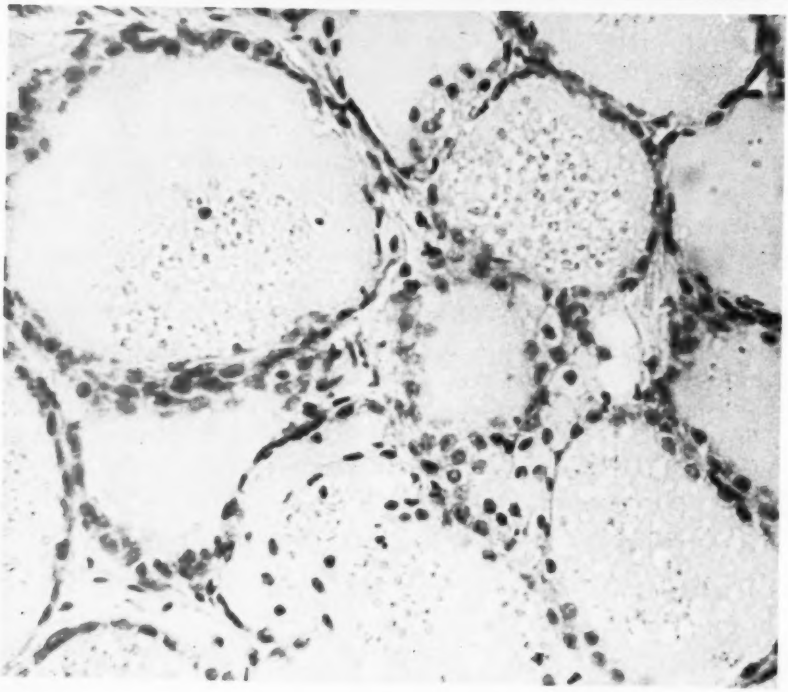
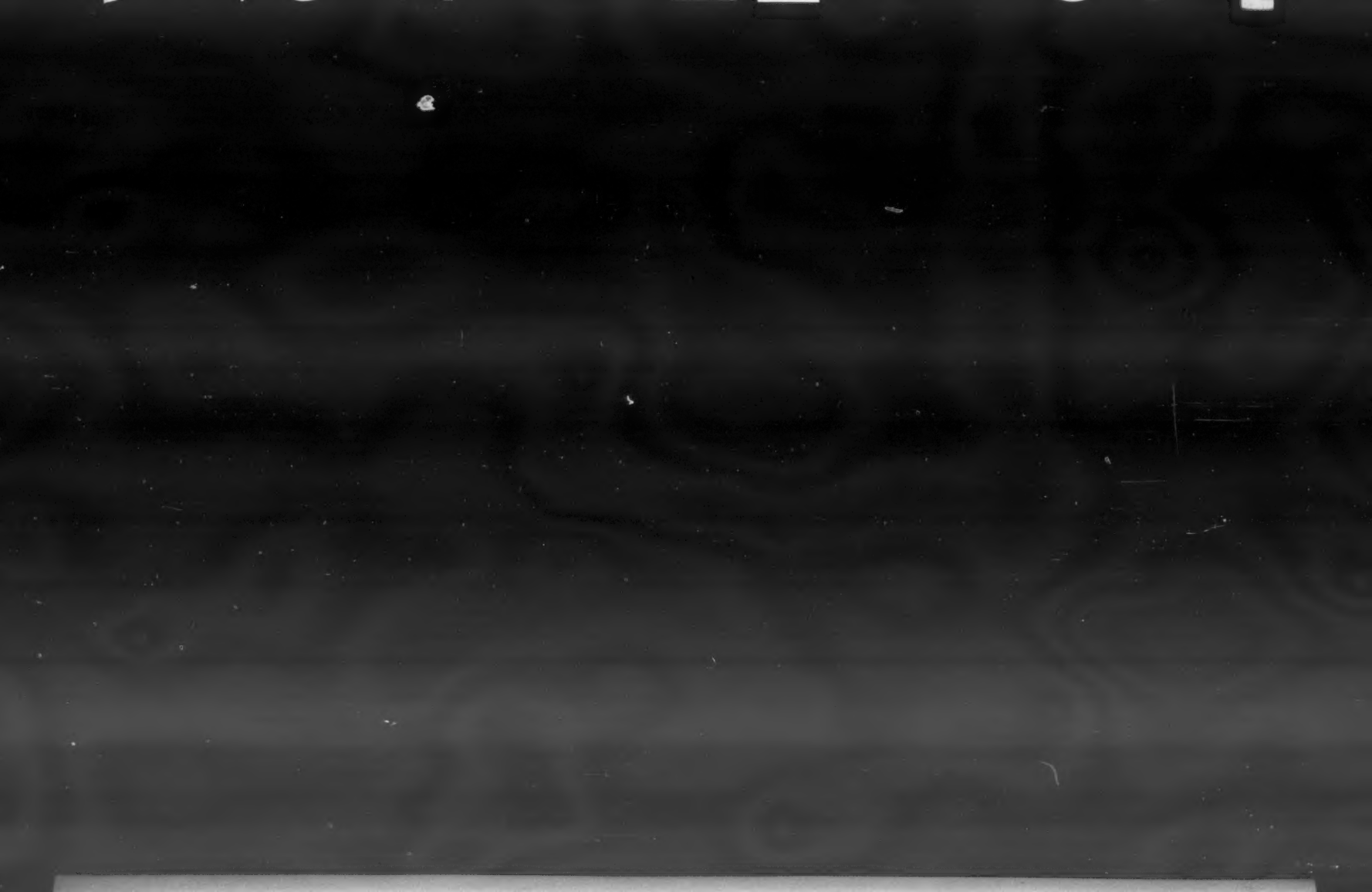
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